Biphenyl Synthase from Gentian and Pear

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Abstract

Gentiana lutea (Gentianaceae) is a herbaceous medicinal plant containing amarogentin, while pears (*Pyrus* sp., Rosaceae) are important fruit trees and cultivated in all temperate-zone countries. Despite their high economic value, their disease resistance mechanisms are poorly understood. Following fungal infection, biphenyls and dibenzofurans are formed as phytoalexins. Biphenylcarboxylate synthase (BICS) from gentian and biphenyl synthase (BIS) from pear are related plant-specific PKSs that are the key enzymes of the biosynthetic pathways of amarogentin and Maloideae-specific phytoalexins, respectively. Two cDNA fragments were obtained from *G. lutea.* The clones were 1343 and 741 bp long and shared 60.8, 51.8 % identity, respectively with chalcone synthase (Ms, CHS) but lacked appreciable identity with BIS and benzophenone synthase (BPS). In addition, two BIS cDNAs cloned from young leaves of *P. communis* were functionally characterized. Both enzymes preferred benzoyl-CoA (K_m 1.6 for BIS1 and 1.7 for BIS2*)*. With salicoyl-CoA, the enzymatic product was 4-hydroxycoumarin. The two recombinant proteins cannot accept both *m*- and *p*-hydroxybenzoyl-CoAs and *p*-coumaroyl-CoA. Expression of the BISs was studied in leaves of *P. communis* after infection with *Erwinia amylovora*. While BIS2 was constitutively expressed, BIS1 was induced after infection. Six residues differ between the BIS1 and BIS2 amino acid sequences and were altered in BIS1 by site-directed mutagenesis. The S275N mutation enhanced the catalytic activity with both benzoyl-CoA and salicoyl-CoA as starters, while the L299F substitution abolished the activity with salicoyl-CoA. The double mutant S275N/L299F exhibited strongly increased activity with both starter substrates.

Zusammenfassung

Gentiana lutea (Gentianaceae) ist eine krautige Heilpflanze enthält amarogentin, während Birnen (*Pyrus* sp., Rosaceae) wichtige Obstbäume und kultiviert werden, in alle Länder gemäßigter Zonen. Trotz ihrer hohen wirtschaftlichen Wert, ihre Resistenz gegen Krankheiten Mechanismen wenig verstanden. Nach Pilzinfektion sind Biphenyle und Dibenzofurane als Phytoalexine gebildet. Biphenylcarboxylate Synthase (BIC) aus Enzian und Biphenyl-Synthase (BIS) aus Birnen sind anlagenspezifische PKS, dass die Schlüsselenzyme der Biosynthese von amarogentin und Maloideae-spezifische Phytoalexine, jeweils verbunden sind. Zwei cDNA-Fragmente wurden von *G. lutea* gewonnen. Die Klone wurden 1343 und 741 bp lang und freigegebenen 60,8, 51,8% Identität, jeweils mit Chalconsynthase (Ms, CHS), aber keine nennenswerte Identität mit BIS und Benzophenon-Synthase (BPS). Darüber hinaus cDNAs zwei BIS aus jungen Blättern von *P. communis* geklont wurden funktionell charakterisiert. Beide Enzyme Benzoyl-CoA (Km 1.6 für BIS1 bevorzugt und 1.7 für BIS2). Mit salicoyl-CoA, wurde die enzymatische Produkt 4-hydroxycoumarin. Die beiden rekombinanten Proteinen kann nicht akzeptieren, auch *m*-und *p*-hydroxybenzoyl-CoA und *p*-Coumaroyl-CoA. Expression des BISs war in Blättern von *P. communis* nach Infektion mit *Erwinia amylovora* untersucht. Während BIS2 konstitutiv exprimiert wurde, war BIS1 nach Infektion induziert. Sechs Rückstände unterscheiden zwischen der BIS1 und BIS2 Aminosäuresequenzen und wurden in BIS1 durch ortsspezifische Mutagenese verändert. Die Mutation S275N verstärkte die katalytische Aktivität sowohl mit benzoyl-CoA und salicoyl-CoA als Starter, während die Substitution L299F schaffte die Aktivität mit salicoyl-CoA. Die Doppel-Mutante S275N/L299F zeigten stark erhöhte Aktivität mit beiden Starter-Substraten.

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Asya Khemaj Swiddan

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Abbreviations

Aa	Amino acid
ACS	Acridone synthase
ALS	Aloesone synthase
APS	Ammonium peroxy disulphate
BAS	Benzalacetone synthase
bp	Base pair
BICS	Biphenylcarboxylate synthase
BIS	Biphenyl synthase
BLAST	Basic Local Alignment Search Tool
BPS	Benzophenone synthase
BSA	Bovine serum albumin
°C	Degree Celsius (centigrade)
cDNA	Complementary deoxyribo nucleic acid
CHS	Chalcone synthase
CTAL	4-coumaroyltriacetic acid lactone
DNA	Deoxyribo nucleic acid
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
DOC	Deoxycholate
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
E. amylovora	*Erwinia amylovora*
g	Gram
G. lutea	*Gentiana lutea*
GSP	Gene specific primer
3GT	3-O-glucosyltransferases
6X His	Hexa histidine tag
HPLC	High performance liquid chromatography
h	Hour
IPTG	Isopropyl- 1 – thio – β – D – galactopyranoside
kDa	Kilo Dalton
K_{cat}	Catalytic constant

K_{cat}/K_m	Ratio catalytic constant / Kinetic constant
K_m	Kinetic constant Michaelis-constant
L	Liter
LB	Luria Bertani
M. domestica	*Malus domestica*
µm	Micrometer
µl	Microliter
mg	Milligram
ml	Milliliter
mM	Millimolar
M	Molar
Min	Minute
mRNA	Messenger RNA
MS	Murashige Skoog
Ni-NTA	Nickel-nitrilotriacetic acid
Nm	Nano meter
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
P. communis	*Pyrus communis*
Pfu-polymerase	Pyrococcus furiosus polymerase
PKSs	Polyketide synthases
PPS	Phenylpyrone synthase
P. pyrifolia	*Pyrus pyrifolia*
2-PS	2-Pyrone synthase
RACE	Rapid amplification of cDNA ends
R. palmatum	*Rheum palmatum*
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT-PCR	Reverse transcription PCR
SDM-PCR	Site directed mutagenesis PCR

SDS-PAGE	SDS-Polyacrylamide – gel electrophoresis
SDS	Sodium dodecyl sulphate
Sec	Second
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
TEMED	N,N,N´,N´-tetramethylethylene – diamine
TCA	Trichloroacetic acide
U	Unit
V	Volt
V/V%	Volume per volume percentage
W/V%	Weight per volume percentage
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
λ	Wavelength

Amino acids:

Abbreviation	Amino acid
A = Ala	Alanine
C = Cys	Cysteine
D = Asp	Aspartic acid
E = Glu	Glutamic acid
F = Phe	Phenylalanine
G = Gly	Glycine
H = His	Histidine
I = Ile	Isoleucine
K = Lys	Lysine
L = Leu	Leucine
M = Met	Methionine
N = Asn	Asparagine
P = Pro	Proline
Q = Gln	Glutamine
R = Arg	Arginine
S = Ser	Serine
T = Thr	Threonine
V = Val	Valine
W = Trp	Tryptophan
Y = Tyr	Tyrosine

01 INTRODUCTION

1.1. Natural products

Plants are fundamental to almost all life on earth, providing substance for organisms ranging from bacteria to large mammals. With their unique capacity for photosynthesis, they form the basis of the biological food chain, meanwhile producing oxygen and mopping up excess levels of the green-house gas carbon dioxide. Plants also perform a number of other important environmental services, recycling essential nutrients, stabilizing soils, protecting water catchment areas and helping to control rainfall via the process of transpiration (Cotton, 1996). Current investigations of medicinal plants and dietary plants play an important role in improving quality of life and human health. Plants synthesize an immense variety of metabolites, which are generally classified into two major groups based on their function. Primary metabolites are essential for growth and universally used, whereas secondary metabolism is highly diverse and variable and plays a role for survival of the producing organism within its natural habitat (Bode and Müller, 2003). Plant secondary metabolites include a vast array of compounds that to date sum up to more than 200,000 defined structures (Hartmann, 2007). These compounds are usually classified into major groups depending on the basic building blocks of the final structures, e.g. the terpenes that are formed from isoprenoid moieties, or the polyketides, that are assembled from acetyl units and a variable starter. Thanks to the improvement of biochemical techniques and the rise of molecular biology, it has been clearly demonstrated that secondary products play a major role in the adaptation of plants to their environment, but also for human health, because almost 50% of the most important medications are based on these so-called natural products (Bode and Müller, 2003). The present work deals with structurally related natural products encountered in the plant families Gentianaceae and Rosaceae.

1.2. Gentianaceae

The plant family Gentianaceae described by Jussieu (1789) comprises 76 genera and about 1200 species (Me´sza´ros et al, 1996), which contain a number of pharmacologically interesting natural products, such as secoiridoids and xanthones. Xanthones are predominant in only two plant families, the Gentianaceae and the Clusiaceae (Guttiferae), where xanthones are well known for their antibacterial, antitumoral and anti-HIV activities. Gentianaceae are rich in highly oxygenated and glycosylated xanthones, whereas Clusiaceae contain prenylated compounds (Bennett and Lee, 1989). The Gentianaceae comprise a number of medicinal plants, such as *Gentiana lutea* and *Swertia chirata*.

1.3. *Gentiana lutea*

Fig.1. *Gentiana lutea*

Gentiana lutea is a perennial herbaceous medicinal plant (Fig. 1). It commonly grows in alpine areas of central and southern Europe and in western Asia, most often at elevations between 600 to 2,500 m. It is widely used in officinal and traditional medicine. The officinal drug Gentianae radix is listed in many pharmacopoeial monographs and plant registers of the world (Nikolaeva et al, 1983, Bruneton, 1995, Menkovic et al, 2000, Jevdjović et al, 2007, Aberham et al, 2007 and Citova et al, 2008).

The name *Gentiana lutea*, is derived from Gentius King of Illyria (west Jugoslavia), who was the first to use it in the second century (Tucakov et al, 1960).

The genus *Gentiana* comprises a number of endangered species, such as *G. cruciata, G. purpurea, G. panonica, G. tibetica, G. kuroo, G. acaulis, G. crispate, G. dinarica, G. nivalis, G. pneumonante, G. utriculosa, G. verna,* and *G. asclepiadea* (Momcilovic et al, 1997 and Tucakov et al, 1960). Due to uncontrolled use for liquor

manufacturing (glucoside extraction from roots), these species are endangered and their protection is very important. This is why the micropropagation of gentian species is of great interest, however, attempts to cultivate gentian were met with difficulties and finally proved to be uneconomic (Jevdjović et al, 2007, Momcilovic et al, 1997and Tucakov et al, 1960).

1.3.1. Plant Description

Yellow gentian is a tall and hardy herb (1 – 1.5 m height), which contains leaves with parallel venation, broad, opposite and decussate, sheathing at the top of the stem (Fig. 2). The yellow flowers are pseudowhorled at the base of the leaves.

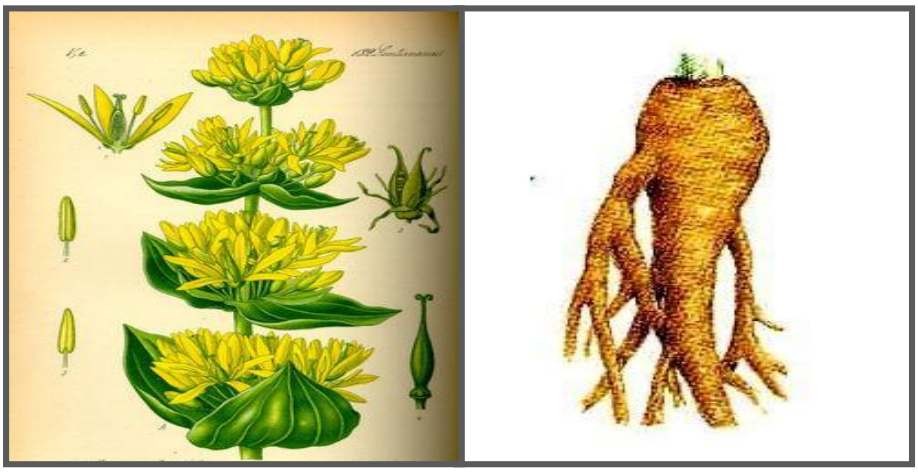


Fig. 2. Gentian flower and root (from Flora of Germany, Austria and Switzerland 1889)

The drug consists of wrinkled, hard, and hardy roots (10 – 40 mm in diameter) which are brittle and break with a short, more or less reddish-yellow fracture (Fig. 2; Bruneton, 1995, Capasso et al, 2003 and Jevdjović et al, 2007).

1.3.2. Mode of action

Gentian stimulates the taste buds and increases by reflex action the flow of saliva and stomach secretions. The bitter taste of the drug is mainly attributed to amarogentin and amaroswerin which are considered the most bitter substances

known to man. Both have a bitterness value of 58,000,000 compared to only 200,000 achieved by quinine. They are used to some extent as alternative bittering agents to quinine in soft drinks (Capasso et al, 2003, Keil et al, 1999).

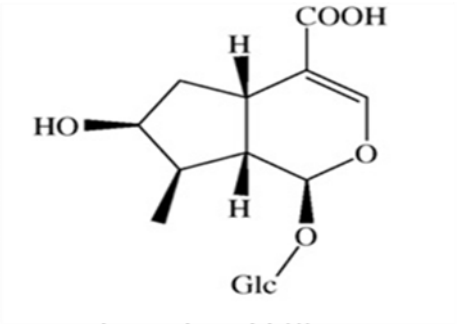
1.3.3. Pharmacological activities of the *G. lutea* constituents

Typical constituents of Gentianaceae are secoiridoids and xanthones. Recently, xanthones with acetylcholinesterase (AChE) inhibitory activity have been isolated (Urbain et al, 2008). In vertebrates and arthropods, acethylcholine (ACh) is one of the major compounds by which electrical impulses carried by nerve cells are transmitted to another nerve cell or to voluntary and involuntary muscles. Two receptor types sensitive to ACh are muscarinic and nicotinic receptors. Muscarinic receptors are chiefly associated with the peripheral nervous system and with smooth and cardiac muscles, while the nicotinic receptors are found in the central nervous system (CNS) and in the motor end plates which are the synapses between nerves and skeletal muscles. In recent years, AChE has been found to be involved in a number of other functions besides nerve impulse transmission. These include roles as an adhesion protein, a bone matrix protein, and in neurite growth and in the production of amyloid fibrils, which are characteristically found in the brain cells of patients with Alzheimer´s disease. AChE degrades the neurotransmitter acetylcholine and has been shown to accelerate Alzheimer´s disease (Urbain et al, 2008 and Houghton et al, 2006). Inhibition of MAO and subsequent hydrogen peroxide (H_2O_2) generation effectively prevents depression and various oxidative stresses in the brain, because the MAO reaction yields aldehydes and H_2O_2, which induces apoptosis. Thus MAO-inhibting plant extracts could be useful as potential neuroprotectants in the treatment or prevention of depression (Haraguchi et al, 2004). The other gentian constituents and their activities are listed in Table 1.

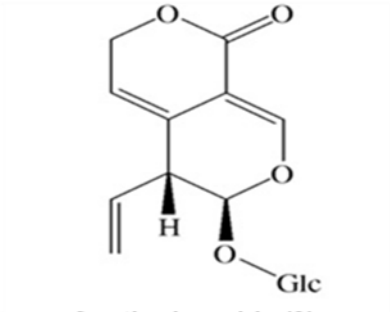
Table 1. Pharmacological activities of the *G. lutea* constituents (Aberham et al, 2007, Recio et al, 1993, Öztürk et al, 2006, Haraguchi et al, 2004, Schmieder et al, 2007 and Capasso et al, 2003)

Constituent (content)	Class[a]	Pharmacological activity
Loganic acid (1) (0.10-0.76%)	Iridoid	Anti-inflammatory (Recio et al, 1993)
Swertiamarin (2) (0.21-0.45%)	Secoiridoid-glycoside	Gastric stimulant, cholagogue, hepatoprotective, wound healing (Öztürk et al, 2006 and Capasso et al, 2003)
Gentiopicroside (3) (4.46-9.53%)		
Amarogentin (4) (≤0.09%)		
Sweroside (5) (trace)		
Gentioside (6) and its isomer (7) (0.03%)	Xanthone glycoside	Potent inhibitor of MAO type A and B *in vitro* (Haraguchi et al, 2004)
Gentisin (8) (0.02%)	Xanthone	Potent inhibitor of MAO type A and B *in vitro* (Haraguchi et al, 2004)
Isogentisin (9) (0.11%)		Potent inhibitor of MAO type A and B *in vitro*, protection from endothelial damage caused by cigarette smoking. (Haraguchi et al, 2004 and Schmieder et al, 2007

[a]see Fig. 3 for chemical structures.

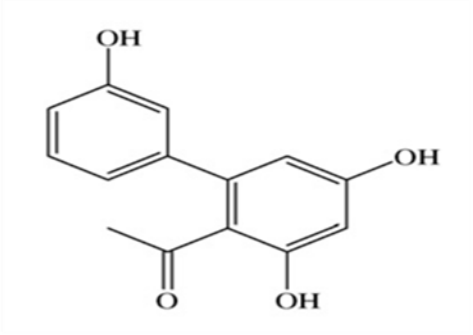


Loganic acid (1)

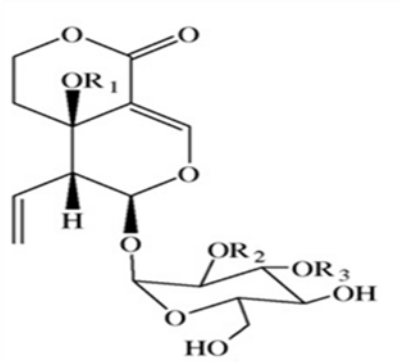


Gentiopicroside (3)

	R1	R2	R3
Swertiamarine(2)	H	H	H
Amarogentin (4)	H	A	H
Sweroside (5)	H	H	H



Residue A



	R1	R2	R3
Gentioside (5)	CH_3	H	*Prim*
Gentioside isomer (6)	*Prim*	H	CH_3
Gentisin (7)	H	H	CH_3
Isogentisin (8)	CH_3	H	H

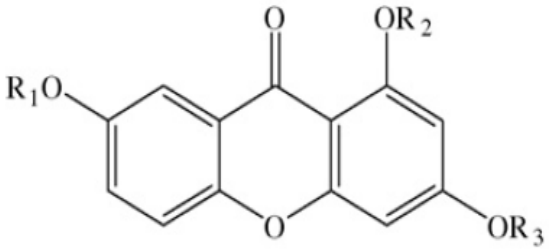


Fig. 3. Chemical structures of the *Gentiana lutea* constituents

1.3.4. Amarogentin

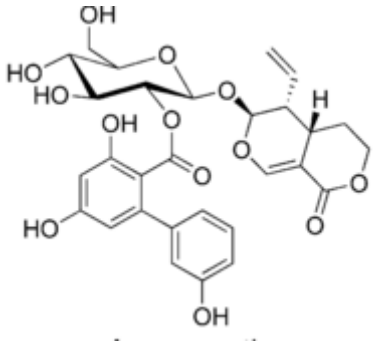


Fig. 4. Amarogentin

Amarogentin is a monoterpene glucoside derivative consisting of a secoiridoid glucoside attached to an unusual 3,3´,5-trihydroxybiphenyl-2-carboxylic acid moiety (Fig. 4). A retrobiosynthetic ^{13}C NMR study demonstrated that the sweroside moiety is formed from DMAPP and IPP as building blocks, which are predominantly derived from the deoxyxylulose phosphate pathway (Wang et al, 2001). A chemical synthesis of the biphenyl carboxylate moiety was reported (Wang et al, 2000). In recent years, interesting novel pharmacological activities of amarogentin have been observed. The compound is a potent inhibitor of DNA topoisomerase I from *Leishmania donovani* (Ray et al, 1996). Human leishmaniasis is a widespread parasitic disease caused by *Leishmania* species and is estimated to affect around 12 million people worldwide (Kedyierski et al, 2009).Topoisomerase I is involved in important cellular processes, such as DNA replication, transcription and repair. In a hamster model of experimental leishmaniasis, amarogentin lacked acute toxicity (Medda et al, 1999; Carvalho and Ferreira, 2001) and thus might be a lead compound for designing effective antileishmanial drugs. In addition, amarogentin exhibits anti-tumoral activity. An extract from *Swertia chirata* highly enriched in amarogentin significantly inhibited the proliferation of cancer cells and induced apoptosis in a mouse skin carcinogenesis model (Saha et al, 2006).

The biphenylcarboxylate moiety of amarogentin is likely to be biosynthesized via the polyketide pathway from one molecule of 3-hydroxybenzoyl-CoA and three molecules of malonyl-CoA (Fig. 5). The enzyme that probably catalyzes this reaction is called biphenyl carboxylate synthase (BICS).

In contrast, biphenyl synthase (BIS) is responsible for the formation of biphenyls lacking the 2-carboxyl group and has already been studied (Liu et al, 2004; 2007). Biphenyls are phytoalexins of the Rosaceous subfamily Maloideae, which includes important fruit trees such as apple and pear.

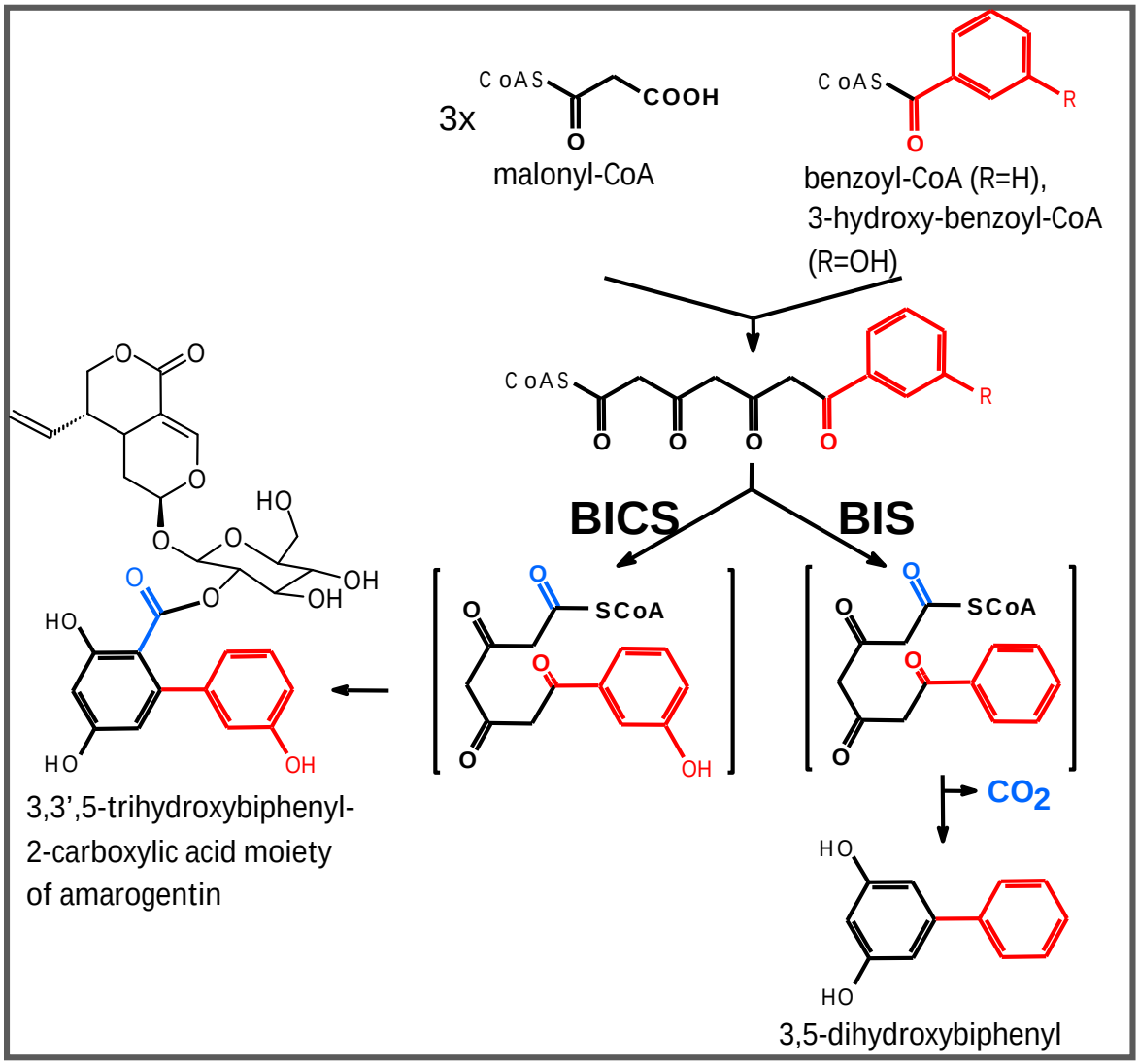


Fig. 5. Established reaction of biphenyl synthase (BIS) (Liu et al, 2007) and proposed reaction of biphenylcarboxylate synthase (BICS)

1.4. Rosaceae (sub-family Maloideae)

Rosaceae is a large family of approximately 122 genera and 3.370 species of trees, shrubs and herbs of worldwide distribution with abundance in north temperate regions (Devore et al, 2007). The name is derived from the genus *Rosa*. The largest genera are *Sorbus*, *Crataegus* and *Cotoneaster*. The family includes many important fruit crops such as apple (*Malus domestica*), peach (*Prunus persica*), plum (*Prunus domestica* and *P. salicina*), cherry (*Prunus avium* and *P. cerasus*), apricot (*Prunus armeniaca*), almond (*Prunus dulcis*), pear (*Pyrus communis*), quince (*Cydonia oblonga*), strawberry (*Fragaria ananassa*), rose (*Rosa* sp.) and loquat (*Eriobotrya japonica*). Furthermore, hawthorn (*Crataegus* sp.) and mountain ash (*Sorbus aucuparia*) are grown as ornamental trees. Most of these species are woody perennials with a long intergeneration period due to their juvenile phase and large plant sizes (Dirlewanger et al, 2004).The Maloideae accumulate biphenyls and dibenzofurans as phytoalexins in the sapwood (Kokubun and Harborne 1995). Interestingly, other Rosaceous subfamilies, such as Prunoideae and Spiraeoideae fail to produce these phytoalexins. Although the Maloideae have considerable economic importance, their disease resistance mechanisms are relatively poorly understood.

1.5. Pear species

Pear species are important pome fruits because they are favorable foodstuff due to their delicious flavor and manifold cultivars, which are grown in all temperate-zone countries. The world production of pear fruits is about 17 million tons a year. Present-day, pear cultivation is continuously rising worldwide and drastically expanding in Asia (Fischer et al, 2007). According to the United State Department of Agriculture (USDA), the global pear production will continue to increase. In the major reporting countries, it was estimated to rise in the marketing years 2005/2006 for the 10th consecutive season (Fig. 6).

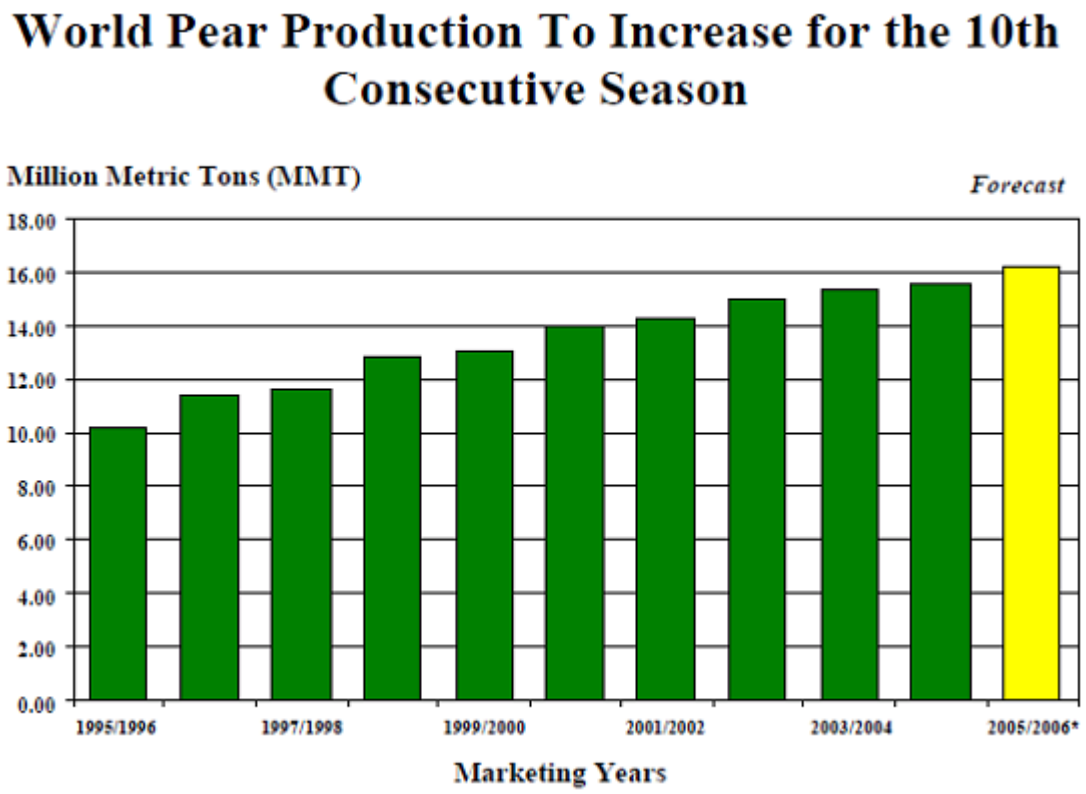


Fig. 6. Increase in world pear production from 1995 – 2006. (Source : United States Department of Agriculture (USDA)

1.5.1. Plant description

Pear is a well-known tree that grows reaching 5 m height (Fig. 8). The leaves are alternate, deciduous, oval, glabrous with long petioles and shiny with wavy, slightly-tooted margins. Flowering occurs early in the spring before the leaves emerge. The flowers are wide and white in bunches. The pear fruit is sweet juicy yellow or green or brown or combination of these colors with a rounded shape that becomes narrower towards the stalk (Kotb, 1985).

1.5.2. Active constituents

Pears contain a broad spectrum of phenolic compounds comprising various flavonoid classes (anthocyanins, flavonols, monomeric catechins), polymeric flavan-3-ols

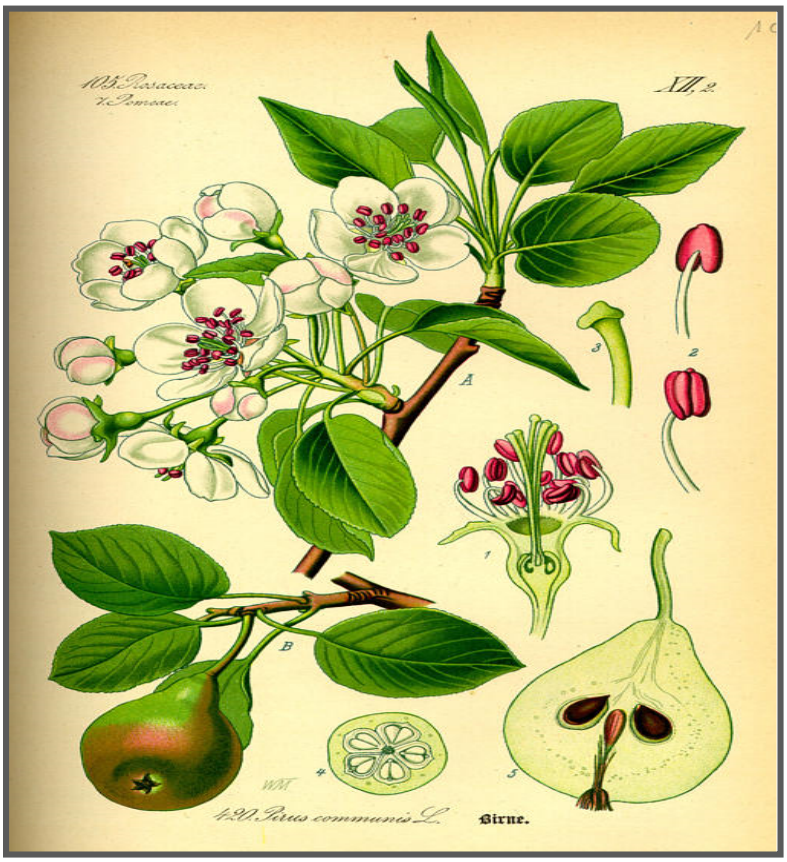


Fig. 7. *Pyrus communis* (from Flora of Germany and Switzerland, 1885)

(proanthocyanidins, syn. condensed tannins), flavanones, hydroxyphenolic acids (mostly hydroxycinnamic acids derived from caffeic acid and *p*-coumaric acids), and the *p*-hydroquinone glucoside arbutin. Leaves contain arbutin, its derivatives, and hydroxycinnamic acids. Among flavonoids of leaves and fruits, B-ring dihydroxylated flavonol derivates (quercetin and isorhamnetin) and monomeric and polymeric flavan-3-ols (epicathechin and proanthocyanidins) are dominant. These compounds contribute to color and to pathgen resistance. The bark of the root contains tannins and phlorhizin. The fruit is rich in pectin and protein. Dominant polyphenols in fruits are hydroxycinnamic acids, and seeds contain amygdalin. Decoction of leaves is used to relax cramps, while fruits are hypotensive, and indicated in case of anemia and diarrhea (Kotb, 1985 and Fischer et al, 2007). The phytoalexins of pear belong to biphenyls and dibenzofurans (Kokubun and Harborne, 1995).

1.5.3. Biphenyls and dibenzofurans

The homodimeric enzyme BIS catalyzes the formation of the C_{12} skeleton of biphenyls and dibenzofurans (Liu et al, 2004). The preferred starter substrate for BIS is benzoyl-CoA that is a rare starter molecule for PKSs. The first plant PKS found to use benzoyl-CoA as starter substrate was benzophenone synthase (BPS), which is the key enzyme of benzophenone and xanthone metabolism (Liu et al., 2003).

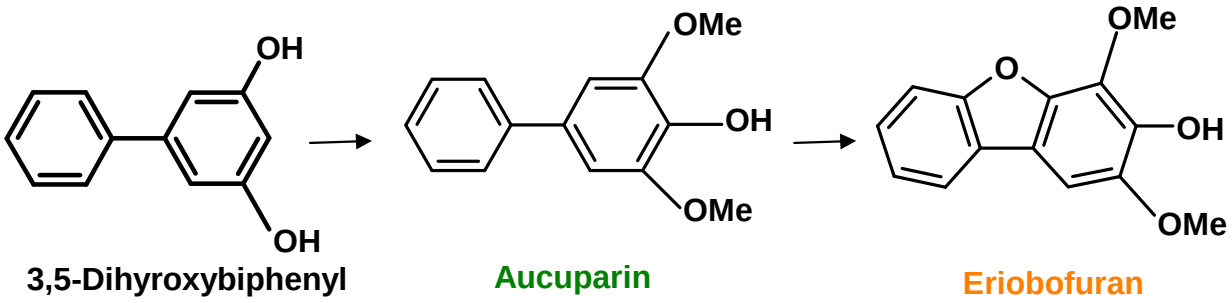


Fig. 8. Proposed biosynthesis of aucuparin and eriobofuran

Like BPS, BIS catalyzes the iterative condensation of benzoyl-CoA with three acetyl units from malonyl-CoA to give an identical linear tetraketide. In the active site cavity of BIS, this intermediate, then undergos an intramolecular C2 ⟶ C7 aldol condensation and decarboxylative elimination of the terminal carboxyl group to form 3,5-dihydroxybiphenyl (Fig. 5). Further yet undetected reactions convert the BIS product into phytoalexins (Fig. 8). The ability to produce these two classes of inducible defence compounds is confined to Maloideae (Kokubun and Harborne, 1995). The phytoalexins were not found in leaves, with the exception of *Sorbus aucuparia* leaves that accumulated the biphenyl aucuparin in response to biotic and abiotic elicitation (Kokubun and Harborne, 1994). The antifungal activity of biphenyls and dibenzofurans is attributed to both inhibition of spore germination and repression of mycelial growth at concentrations thought to be present at localized infections sites (Hrazdina 2003). Formation of biphenyls and dibenzofurans thus appears to provide one pathogen defence strategy of Maloideae (Dixon 2001).

1.6. Research strategies and objectives

BICS from gentian and BIS from pear are related plant-specific PKSs that are the key enzymes of the biosynthetic pathways of amarogentin and Maloideae-specific phytoalexins, respectively. The aim of this study was molecular cloning of a BICS-cDNA and functional expression of the recombinant enzyme in *E. coli.* Furthermore, BISs from pear were heterologously expressed to allow for a comparative characterization of the affinity-purified enzymes. In addition, structure-function relationships were studied using site-directed mutagenesis. The resulting enzyme mutants were characterized with respect to changes in substrate and product specificities.

02 MATERIALS AND METHODS

2.1. Plant material

2.1.1. *Gentiana lutea*

Gentiana lutea (Gentianaceae) was obtained as mature plant from Rühlemann`s Kräuter und Duftpflanzen. It was grown and maintained in the greenhouse of the institute. Roots and leaves were collected separately for nucleic acid separation and frozen at -20°C until used. In vitro shoots were grown on MS medium (Murashige and Skoog, 1962). For aseptic cultivation, shoots were sterilized in 6% NaOCl for 5 minutes followed by three times washes in sterile deionized water.

2.1.2. Pear species

Pyrus communis cultivar 'Conference' (Rosaceae, sub family Maloideae,) was obtained as mature plant from the institute for Resistance Research and Stress Tolerance, Quedlinburg (Julius Kühn Institute, Federal Research Centre for Cultivated Plants). Leaves of Pyrus *pyrifolia* were obtained from the botanical garden of the TU Braunschweig, frozen immediately in liquid N_2 and stored at -20°C until used.

2.2. Chemicals

Commonly used chemicals and gases were purchased at standard grade from the following companies:

Aldrich	Fluka	Riedel-deHaën
Applichem	Merck	Serva
Bio-rad	Roth	Sigma

Aqueous solutions were prepared with milli-Q water which was purified by using the Milli-Q water purification system (Millipore) and an Arium 611 VF water purification system (Sartorius, Germany).

2.2.1. Media and components

2.2.1.1. Luria-Bertani (LB) medium

LB medium was used as an incubation medium for growing *E. coli* bacteria at 37°C.

Peptone 10 g	Roth	Distilled water up to 1000 ml, adjust pH to 7.0 with NaOH, autoclave
Sodium chloride 10 g	Roth	
Yeast extract 5 g	Roth	

2.2.1.2. LB agar plates

§ Containing ampicillin and chloramphenicol, they were used as a growth medium for bacteria (*E. coli [BL 21]*). LB medium that contained in addition:

Agar 1.5%	Roth	To solidify LB medium
Ampicillin 100 mg / ml	Roth	Dissolve 1 g ampicillin in 10 ml distilled water and, filter-sterilize through a 0.22 µm filter
Chloramphenicol 30 mg / ml	Fluka	Dissolve 0.3 g chloramphenicol in 10 ml absolute ethanol

§ Containing ampicillin, IPTG and X-Gal (for white/blue colony selection), they were used as a growth medium for bacteria (*E. coli* [DH5α]). LB medium that contained in addition:

Agar 1.5 %	Roth	
Ampicillin 100 mg / ml	Roth	
IPTG 0.5 M	Applichem	Dissolve 1.2 g IPTG in 10 ml distilled water and filter-sterilize through a 0.22 µm filter
X-Gal 40 mg / ml	Sigma Aldrich	Dissolve 0.4 g X-Gal in 10 ml *N´-N´*-dimethylformamide (Fluka)

2.2.1.3. Murashige Skoog (MS) medium (Murashige and Skoog, 1962)

The MS medium is used as an artificial medium for in vitro tissue cultures. Stock solutions (20 fold) were prepared for microelements, vitamins and macro- elements, which were diluted 20 times to give the final concentrations as follows:

1. Microelements:

H_3BO_3	6.2 mg	Roth
$MnSO_4 . H_2O$	16.9 mg	Fluka
$ZnSO_4 . 7H_2O$	10.6 mg	E. Merck
KI	0.83 mg	Sigma
$Na_2MoO_4 . 2 H_2O$	0.25 mg	Merck

$CuSO_4 . 5 H_2O$	0.025 mg	Merck
$CoCL_2 . 6 H_2O$	0.025 mg	E. Merck
$Na_2EDTA . 2 H_2O$	37.2 mg	Roth
$FeSO_4 . 7 H_2O$	27.5 mg	Merck

2. Vitamins:

Glycin	2.0 mg	Roth
Myo-Inositol	0.1 mg	Sigma
Nicotinic acid	0.5 mg	Merck
Pyridoxine-HCL	0.5 mg	E. Merck
Thiamin- HCL	0.1 mg	Serva

3. Macroelements:

NH_4NO_3	1.65 g	Merck
KNO_3	1.9 g	Fluka
$CaCL_2 . 2 H_2O$	0.44 g	Merck
$MgSO_4 . 7 H_2O$	0.37 g	Merck
KH_2PO_4	0.17 g	Merck

4. Carbohydrates:

Agar-agar	8 g	Roth
Sucrose	20 g	Serva

Mix with 800 ml distilled water, adjust the pH to 5.8 with 5 N NaOH and the final volume to 1 liter and autoclave the medium for sterilization.

2.2.1.4. Soc medium

This is a nutrient-rich medium which is widely used for transformation of *E. coli* competent cells to maximize the transformation efficiency.

§ Mg^{2+} solution 2 M

$MgCl_2 . 6H_2O$	20.33 g	Calbiochem	Dissolve in 100 ml water and subject to sterile filtration
$MgSO_4 . 7H_2O$	24.65 g	Riedel-deHaën	

Peptone	2 g	Roth
Yeast extract	0.5 g	Roth
NaCl	1 M	Roth
KCl	1 M	Roth

Dissolve in 97 ml distilled water, autoclave, cool and then add:

Mg^{2+} solution	2 M	1 ml
Glucose solution	2 M	1 ml

Distilled water up to 100 ml and store at - 20°C

2.2.2. Enzymes

2.2.2.1. Reverse transcription

M-MWLV Reverse transcriptase	Fermentas
M-MWLV-RT 5X reaction buffer	Fermentas
Ribolock RNase inhibitor	Fermentas
PrimeScript™Reverse Transcriptase	Takara Bio INC. (Clontech.)

2.2.2.2. DNA Polymerases

Taq DNA polymerase	Fermentas
Pfu DNA polymerase	Fermentas
Dream Taq DNA polymerase	Fermentas

2.2.2.3. Restriction enzymes

EcoRI	Fermentas
DpnI	Fermentas

2.2.2.4. Other enzymes

RNase A	Fermentas
T4 DNA ligase	Fermentas
RNase T	Fermentas

2.2.3. Primers

All primers were synthesized in HPSF quality by MWG-Biotech AG (Ebersberg).

A. SMART-RACE and Vector Primers

SMART II	5`-AAG CAG TGG TAT CAA CGC AGA GTA CGC GGG-3`
5`CDS	5`-$(T)_{25}$V N-3`
3`CDS	5`-AAG CAG TGG TAT CAA CGC AGA GTAC$(T)_{30}$V N-3`
RACE-short	5`-CTA ATA CGA CTC ACT ATA GGG C-3`
RACE-long	5`-CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA ACA ACG CAG AGT-3`
RACE-nested	5`-AAG CAG TGG TAA CAA CGC AGA GT-3`
T7	5`-GAA TTG TAA TAC GAC TCA CTA TAG-3`
SP6	5`-GAT TTA GGT GAC ACT ATA GAA TAC-3`

B. Universal primers used in restriction site PCR

PF1(EcoRI) RSO	5`-GTA ATA CGA CTC ACT ATA GGG AATTC-3`
PF2 (TATA box)	5`-GTA ATA CGA CTC ACT ATA GGY RSM TAT AWA-3`

C. Universal primers used in TATA protocol

TATA primer	5`-GAT TCT AGA (CT) (CT)5 CTA TA(AT) A(AT)A (GC) (AC)-`3

D. BICS Degenerate primers

5CHS dgsense	T_m57.9°C	5`-ATG GTN CAN GTN GAR GAR GTN-3`
5BPS dgsense	T_m60.8°C	5`-ATG GCN CCN GCN ATG GAR TAY-3`
Pd5 dgsense	T_m59.0°C	5`-YW5 GG5 TGY KH5 GC5 GG5 GG-3`
Pd1 dgsense	T_m55.9°C	5`-GC5 AT(ACT) AA(AG) GA(AG) TGG GG5 CA-3`
Pd3 dganti	T_m60.6°C	5`-CC5 CC5 GG(AG) TG5 GC(AGT) ATC C-3`
Pd4 dganti	T_m52.4°C	5`-AA5 CC(AG) AA5 A(AG)5 AC5 CCC C-3`
Pd6 dganti	T_m59.4°C	5`-AT5 CS5 SK5 CC5 CC5 GGR TG-3`
Pc1 dgsense	T_m69.3°C	5`-GAG GAA CTG TTC TGA GAC TTG CTA A(AG)G A(CT)(ACT) T(AGCT) C-3`
Pc2 dganti	T_m68.8°C	5`-CAG AAC TCC CCA ATC AAA TCC (CT)TC (AGCT)CC (AGCT)GT-3`

E. BICS gene specific primers

P1 F1 sense	T_m63.1°C	5`- AGT ATT CTG CAC AAC AGG TGG TGT G -`3
P2 F1 anti	T_m63.5°C	5`- TGA GTT ATT TTA GAA ATC GGC TGC C -`3

P3 F1 sense	T_m61.0°C	5`- CAA GGA CTT AGC CGA GAA CAA CAA -`3
P4 F1 anti	T_m63.0°C	5`- ATC TCT TTA CTG ACG GAC AAA GGC C -`3
P5 F1 anti	T_m64.6°C	5`- CCA CAC CAC CTG TTG TGC AGA ATA C -`3
P6 F1 anti	T_m62.2°C	5`- GCA AAA CAA CCT TGG TGG CTA ATC -`3
P7 F1 anti	T_m61.3°C	5`- CTT CTG TAA GAC ACA CAT GTA TCG CTG -`3
P8 F1 anti	T_m64.6°C	5`- CGG AGC CCA TGT ATG CAC TCA CAT TTC -`3
P9 F1 anti	T_m63.7°C	5`- CTA AAT ATT GAA ACT GCG TAG AGC CAC C -`3
P1 F2 sense	T_m58.9°C	5`-CAA AGG TGC ACG AGT TCT-3`
P2 F2 sense	T_m61.0°C	5`-CAG ATT TAG ATG GTC TAG-3`
P3 F2 anti	T_m60.6°C	5`-CAA TCA GTA ATC CCC AGA GGT TG-3`
P4 F2 anti	T_m62.7°C	5`-CTT AGC TTG ACA ATA AGG CCA GCC-3`

F. BIS1 mutation primers

BIS1 R231S sense	T_m72.4°C	5`-CAG AGC CTG AAA TTG AGA GTC CGC TGT TTG AAA TTG TGG CA-3`
BIS1 R231S anti	T_m72.4°C	5`-TGC CAC AAT TTC AAA CAG CGG ACT CTC AAT TTC AGG CTC TG-3`
BIS1 D267E sense	T_m69.4°C	5`-T TAT TAT TTA TCG GGA GAG GTT CCC AAA TTT GTT GGT GGA AG-3`
BIS1 D267E anti	T_m69.4°C	5`-CT TCC ACC AAC AAA TTT GGG AAC CTC TCC CGA TAA ATA ATA A-3`
BIS1 S275N sense	T_m67.6°C	5`-AAA TTT GTT GGT GGA AAT GTT GTGGAT TTT CTG ACT AAA ACT TTT G-3`
BIS1 S275N anti	T_m67.5°C	5`-GTT TTA GTC AGA AAA TCC ACA ACA TTT CCA CCA ACA AAT TTG-3`
BIS1 L299F sense	T_m73.0°C	5`-GAA TAA GGA CTG GAA TTC CTT ATT TTT TAG TGT GCA CCC TGG TGG G-3`

BIS1 L299F anti	T_m72.8°C	5`-CCA CCA GGG TGC ACA CTA AAA AATAAG GAA TTC CAG TCC TTA TTC TTT C-3`
BIS1 R231A sense	T_m73.6°C	5`-AGA GCC TGA AAT TGA GGC GCC GCT GTT TGA AAT TGT GGC A-3`
BIS1 R231A anti	T_m73.8°C	5`-GCC ACA ATT TCA AAC AGC GGC GCC TCA ATT TCA GGC TC-3`
BIS1 R231K sense	T_m69.5°C	5`-GAG CCT GAA ATT GAG AAG CCG CTG TTT GAA ATT GTG-3`
BIS1 R231K anti	T_m69.5°C	5`-CAC AAT TTC AAA CAG CGG CTT CTC AAT TTC AGG CTC-3`
BIS1 R231T sense	T_m70.6°C	5`-CAG AGC CTG AAA TTG AGA CGC CGC TGT TTG AAA TTG-3`
BIS1 R231T anti	T_m72.6°C	5`-CAC AAT TTC AAA CAG CGG CGT CTC AAT TTC AGG CTC TGG-3`

G. BISs gene expression primers

Exp5`pyBIS1	T_m 62.7°C	5`-GAG AGG CCG CTG TTT GAA ATT GTG-3`
Exp3`pyBIS1	T_m61.9°C	5`-CTT TGC CTT CCC CAA TCG ATT TAT TTC-3`
Exp5`pyBIS2	T_m63.2°C	5`-GAG AGT CCA TTG TTT GAA ATC TGG C-3`
Exp3`pyBIS2	T_m63.4°C	5`-CTT TGC CTT CCT CAA TCG ACT TCT TTC-3`

2.2.4. Gel electrophoresis

2.2.4.1. DNA electrophoresis

Agarose gels are used to electrophorese nucleic acid molecules from as small as 50 bases to more than 20 kb, and to determine the size of the separated fragments by comparison to DNA of known length. Usually, 0.8 g of agarose was added to 80 ml of 50x TAE buffer and boiled in the microwave to dissolve the agarose, then cooled down to about 60°C before adding 3 µl of ethidium bromide (1%). Ethidium bromide is an interchelating agent that fluoresces under UV light.

This 1% (w/v) agarose gel was poured into a horizontal gel tray (Biorad), plastic combs with teeth of different width were used to create sample pockets of desired volume and allowed to solidify at room temperature. Samples were mixed with 6x loading buffer (see 2.2.5.2.A) and pipetted into the sample wells. Gels were run in 1x TAE buffer at different voltage values (100-140 mV). A multiimage^{TM}light cabinet (Biozyme) was used to visualize and photograph the gel under UV-light.

Agarose, NEEO ultra quality	Roth
6x loading buffer	Fermentas
DNA Ladder Mix	Fermentas
Ethidiumbromide 1% (10 mg/ml)	Roth

2.2.4.2. Protein electrophoresis (SDS-PAGE)

This is a method for separating proteins according to molecular weight (Laemmli, 1970). The proteins are denatured and rendered monomeric by boiling in the presence of reducing agents (β-mercaptoethanol or dithiotheitol) and negatively charged detergent (SDS). The proteins, which normally differ according to their charges, are all coated with the SDS molecules, which are negatively charged. Hence, all the proteins in the sample become negatively charged and achieve a constant charge to mass ratio. In this way, the separation is only according to the size of the proteins. The final gel for SDS-PAGE consists of two gels; firstly, a 12 % separating gel was poured. In order to achieve a smooth boundary between separating and stacking gel, the separating gel was covered with a layer of water. After polymerization of the separating gel, a 4 % stacking gel was poured over it. Protein samples to be analyzed were mixed with sample buffer (see 2.2.5.2.B) (ratio 5:1) and denatured at 95°C for 5 min. The prepared samples were pipetted into the individual wells of the collecting gel including known proteins. The gel was run at 35 mA/200 V max supplied by a Standard Power Pack P25 (Biometra). The gel was run until the dye front reached the bottom (about 3 hrs). After electrophoresis, the protein bands of interest were located in the gel by using staining techniques.

Item	Separating gel 12 % [ml]	Stacking gel 4% [ml]	Source
H_2O	3.3 ml	3.4 ml	
Acrylamide/Bisacrylamide 30%	4.0 ml	0.83 ml	Bio-Rad
Tris-HCl	2.5 ml (1.5 M) pH 8.8	0.63 ml (0.5 M) pH 6.8	Biosolve LTD
Sodium dodecyl sulphate (SDS) 10 %	100 µl	50 µl	Roth
Ammonium persulphate (APS) 10 %	100 µl	50 µl	Bio-Rad
*N,N,N´,N´*tetramethylethylene-diamine (TEMED)	4 µl	5 µl	Bio-Rad
Unstained protein molecular weight marker, 14,4 kDa to 116 kDa	10 µl		Fermentas

2.2.5. Solutions and buffers

2.2.5.1. Enzyme purification

Constituents		Lysis buffer	Washing buffer	Elution buffer
NaH_2PO_4 (Merck)	⇨	50 mM	50 mM	50 mM
NaCl (Roth)	⇨	30 mM	1.5 M	300 mM
Imidazole (Roth)	⇨	20 mM	50 mM	250 mM

The pH was adjusted with 5 N NaOH to pH 8 and stored at 4°C.

Tris Buffer 50 mM pH 7.5 is used to desalt the soluble protein.

2.2.5.2. Enzyme incubation

KH_2PO_4 buffer 0.1%, the pH was adjusted with concentrated NaOH, autoclaved and stored at room temperature.

2.2.5.3. Enzyme extraction

Ethyl acetate	Roth
Acetic acid 50%	Scientific Fisher

2.2.5.4. Protein Concentration and quantification

Bradford-dye solution	Dissolve 100 mg Coomassie®-brilliant Blue G- 250 in 50 ml ethanol (60 %), add 100 ml *ortho*-phosphoric acid (85 %) and complete the volume to 1 L with distilled water, store at 4°C and filter before use.
DOC solution	Dissolve 0.1 % (w/v) sodium deoxycholic acid and 0.02 % sodium azide in distilled water.
TCA solution	Dissolve 55 % (w/v) trichloroacetic acid in distilled water.

2.2.5.5. Gel electrophoresis

A. DNA electrophoresis

50 X TAE buffer

Tris-HCL	2 M	Biosolve LTD
EDTA	0.05 M	Roth

The pH was adjusted to pH 8.0 with glacial acetic acid

DNA loading dye

Xylencyanol	0.25 g	Sigma
Bromophenol blue	0.25 g	Aldrich
Ficoll 400	25.0 g	Sigma
EDTA	1.46 g	Roth
H_2O	up to 100 ml	

The solution was filtered and sterilized by autoclaving

B. Protein electrophoresis (SDS-PAGE)

Name	Preparation
1.5 M Tris-HCL solution	⇨ Dissolve 18.2 g Tris-HCL in 80 ml of H_2O, the pH was adjusted to pH 8.8 with HCL, and add H_2O to a final volume of 100 ml.
10 % SDS	⇨ Dissolve 10 g SDS in 60 ml of H_2O and add H_2O to a final volume of 100 ml.
10 % APS	⇨ Dissolve 100 mg APS in 1 ml of H_2O (should be freshly prepared).
0.5 M Tris-HCl solution	⇨ Dissolve 6.1 g of Tris-HCL in 80 ml of H_2O, the pH was adjusted to pH 8.8 with HCL, and add H_2O to a final volume of 100 ml.
Electrode buffer 5X pH 8.3	⇨ 0.025 M Tris, 0.192 M glycine, 0.1 % (w/v) SDS, the pH was adjusted to pH 8.3, and add H_2O to a final volume of 1000 ml.
Sample buffer 5X	⇨ 2.13 ml 0.5 M Tris-HCl pH 6.8, 1 g SDS, 5 ml glycerol, 2.56 ml *β*-mercaptoethanol, one spatula tip of bromophenol blue.
Coomassie blue stain	⇨ Dissolve 1.0 g Coomassie®brilliant Blue in 500 ml methanol, 75 ml acetic acid and add distilled water to a final volume of 1000 ml, stir overnight, filtrate before use, and store in a dark bottle.
Destaining solution	⇨ Mix 200 ml methanol and 76 ml acetic acid, complete volume with distilled water to 1000 ml.
Silver stain	
I. Fixing solution (40-60 min)	⇨ Mix 30 % (v/v) ethanol and 10 % (v/v) acetic acid with distilled water.
II. Sensitizing solution (60 min or over night)	⇨ Dissolve 0.2 % (w/v) sodium thiosulphate and 0.5 M sodium acetate in 30 % (v/v) ethanol and distilled water.

Name	Preparation
III. Washing with H_2O (3 times for 5 min)	
IV. Silver solution freshly prepared (30 min)	⇨ Dissolve 0.2 % (w/v) silver nitrate and 0.01 % (v/v) formaldehyde in distilled water.
V. Washing with H_2O (2 times for 2 min)	
VI. Developing solution freshly prepared (until bands become visible)	⇨ Dissolve 6 % (w/v) sodium carbonate (water-free) and 0.02 % (v/v) formaldehyde in distilled water.
VII. Stop solution, freshly prepared	⇨ Dissolve 1.5 % (w/v) Na_2EDTA in distilled water.

2.2.5.3. Plasmid isolation

All buffers were mixed with distilled water, adjusted to 250 ml and autoclaved.

Buffer P1	Tris-HCL	50 mM	The pH was adjusted to 8.0, RNase A was freshly added before use.
	EDTA	10 mM	
	RNase	10 mg/ml	
Buffer P2	NaOH	0.2 M	
	SDS	1% (w/v)	
Buffer P3	Potassium acetate	2.55 M	The pH was adjusted to 4.8.

2.3. Host cells

E. coli (Stratagene)	Genotype
K12® DH 5α	*F' φ80þlacZ∆M15 end A1 hsdR17(rk-mk+) supE44 thi-1 λ-gyrA96 relA1 ∆(lacZYA-argFV169) deoR*
BL 21	*F⁻, ompT, hsdS (rB⁻, mB⁻), gal, dcm*
BL21(DE3)pLysS	*F⁻, ompT hsdSB (rB⁻ mB⁻) gal dcm (DE3) pLysS (CamR)*

2.4. Plasmids

pGEM-T easy Vector	(Invitrogen)
pRSET B	(Invitrogen)

2.5. Others

Item	**Supplier**
Malonyl-CoA	Sigma
Benzoyl-CoA	Sigma
o-Hydroxybenzoyl-CoA	Synthesized by Dr. B. Liu (our work group)
m-Hydroxybenzoyl-CoA	Synthesized by M. Gaid (our work group)
p-Hydroxybenzoyl-CoA	Synthesized by Dr. B. Liu (our work group)
Cinnamoyl-CoA	Synthesized by M. Gaid (our work group)
Butyryl-CoA	Sigma
Isovaleryl-CoA	Sigma
Acetyl-CoA	Sigma
Isobutyryl-CoA	Sigma
Hexanoyl-CoA	Sigma
n-Octanoyl-CoA	Sigma

2.6. General Methods of Molecular Biology

2.6.1. Isolation of nucleic acids

2.6.1.1. Isolation of RNA

RNeasy Plant Mini Kit Qiagen

The RNeasy Plant Mini Kit (Qiagen) was used for the isolation of total RNA from different plant organs. Freshly harvested plant tissue (100 mg) or frozen (-80°C) samples (100 mg) were homogenized with liquid nitrogen and disrupted immediately in a lysis buffer containing a strong denaturating agent. Following cell lysis, centrifugation through QIAshredder ™ was conducted to remove insoluble material and to reduce the viscosity of the lysate. The cleared lysate was transferred to RNeasy Mini Spin columns in the presence of absolute ethanol which promotes a selective binding of RNA to the silica-gel-based membrane of the RNeasy Mini Spin column. By centrifugation the contaminants including small RNAs were efficiently washed away, high quality RNAs were eluted in RNase-free water and stored at -80°C until used. The RNA concentration was determined as described in 2.6.2.

2.6.1.2. Isolation of Genomic DNA

DNeasy Plant Mini Kit Qiagen

The DNeasy Plant Mini Kit (Qiagen) was used for the isolation of genomic DNA from different plant organs. Plant material (100 mg) was pulverized in liquid nitrogen and lysed in a buffer containing RNase. Following lysis, cell debris and precipitates were removed by centrifugation through a QIAshreder ™ column. Binding buffer and ethanol were added to the clear lysate to allow DNA adsorption on the membrane in presence of high concentration of chaotropic salts, which removed water from hydrated molecules in solution, whereas contaminants such as proteins and polysaccharides were efficiently removed by two wash steps. Pure DNA was eluted in 10 mM Tris-HCL buffer pH 8.0.

2.6.1.3. DNA Purification from Agarose Gels

Different purification kits were used to obtain pure DNA.

Nucleospin®	Macherey-Nagel
Innu(PREP) DOUBLEpure Kit	Analytik Jena Biosolution

After gel electrophoresis (2.2.4.1), a DNA band was excised under UV light, the DNA fragment was adsorbed to a silica gel membrane in the presence of a high concentration of chaotropic salts, the binding mixture was loaded directly into a Nucleospin column, contaminants like salts and soluble macromolecular components were removed away by washing and the DNA was eluted with slightly alkaline buffer (10 mM Tris-HCL pH 8.0).

2.6.1.4. Plasmid isolation (mini preparation)

(Birnboin and Doly, 1979)

A single white colony growing on platted plates was picked up by using toothpicks to inoculate LB medium containing the appropriate antibiotic. The bacterial culture was allowed to grow over night with shaking at 225 rpm at 37°C. On the second day, cells from 5 ml of the bacterial culture were harvested by centrifugation at 5000 rpm for 5 min. The bacterial pellet was resuspended in 300 µl Buffer P1 (see 2.2.5.3) containing 3 µl (100 mg/ml) RNase A, for bacterial lysis. 300 µl Buffer P2 (see 2.2.5.3) was added, mixed by inverting the tube six times and leave it for 5 min at room temperature. 300 µl ice-cooled buffer P3 (see 2.2.5.3) was added, mixed by inverting the tube six times and incubated in ice for 20 min, followed by centrifugation at 14000 rpm for 10 min. The supernatant (800 µl) was extracted twice with chloroform, then 0.7 volumes of isopropanol were added to the clear supernatant, mixed gently and centrifuged at 14000 rpm for 30 min. The pellet was washed with 500 µl 70% ethanol and centrifuged at 14000 rpm for 10 min. The sample was dried, redissolved in 50 µl sterile water and stored at -20°C.

2.6.2. Determination of the concentration of nucleic acids

The concentration of nucleic acids was determined by measuring the absorbance value of the RNA or DNA samples at a wavelength of 260 nm. An Ultraspec 1000 Pharmacia Biotech spectrophotometer was used to determine the concentration of 1:50 diluted RNA or DNA samples in a volume of 0.5 ml in a 10 mm light Patch Quartz cuvette. An absorbance of 1.0 at 260 nm corresponds to 50 µg DNA/ml.

2.6.3. Primer design

2.6.3.1. Degenerate primers design

(Wilks, 1989; Primrose and Twyma, 2003)

When a family of genes from closely related organisms is known, there is a certain chance to isolate the corresponding gene from the genome of another related organism. Degenerate primers can be used in PCR procedures to search for a member of a gene family or homologous genes from different species. Degenerate primers are based on the amino acid sequences of conserved regions of homologous proteins that can be obtained from a data base (Gene Bank). To design forward and reverse PCR primers, two blocks of conserved amino acid residues should be present. The primers would be a set of oligonucleotides which have a number of options at several positions in the sequence (primer degeneracy) so as to allow annealing to and amplification of a variety of related sequences.

2.6.3.2. Gene specific primer design

(Innis and Gelfand, 1990)

A gene specific primer is designed to have a sequence which is the reverse complement of a region of template or target DNA to which the primer anneals. It should contain 17-25 bases in length and a GC content of approximately 50%. Its 3' prime terminus should end in a G or C, or CG or GC, which prevents breathing

" of ends" and increases the efficiency of the primer. Primer self complementarity should be avoided. The annealing temperature must be approximately 5°C lower than the melting temperature.

2.6.4. Reverse transcription

A reverse transcriptase enzyme is known as RNA-dependent DNA polymerase which is used to synthesize the first strand cDNA (cDNA is a strand of DNA complementary to appropriate mRNA) from total RNA, using an oligo-dT primer which annealed to the poly A tail of the mRNA. SMART stands for switching mechanism at 5` end of RNA template.

3` and 5` -Race methods

3`-Race cDNA		5` -Race cDNA	
Total RNA 1 µg	x µl	Total RNA 1 µg	x µl
3` -CDS primer	1 µl	5` -CDS primer	1 µl
RNase inhibitor	0.5 µl	RNase inhibitor	0.5 µl
Nuclease-free water up to	11.5 µl	Smart II oligo primer	1 µl
		Nuclease-free water up to	11.5 µl

The tubes were incubated at 70°C for 5 min. The PCR tubes were placed on ice for 2 min. Then the following components were added to each reaction:

5x reaction buffer	4 µl
200 U/µl reverse transcriptase	1 µl
dNTP mix (10 mM)	2 µl

The contents of the tubes were mixed by pipetting, incubated at 35°C for 5 min, then at 42°C for 90 min, followed by heat inactivation of the enzyme at 70°C for 15 min.

The cDNA sample was either used for further PCR or stored at -20°C. All primers used are listed in (2.2.3.A).

2.6.5. Polymerase chain reaction (PCR)

(Wolfe and Liston 1998)

In the last decades, PCR has rapidly evolved in the molecular biology field. PCR is a primer-mediated enzymatic amplification of specifically cloned or genomic DNA sequences.The “chain reaction" of the technique refers to an increase in the amount of target DNA obtained through successive cycles of amplification. The basic protocol of PCR is as follows. Double stranded DNA (template) is denatured at high temperature to form single strands, short oligonucleotide primers bind at lower annealing temperature to the single strand complementary templates at the ends flanking the targeted sequence, the temperature is raised for synthesis, by primer extension of the target sequences, the newly synthesized double stranded DNA target sequences are denaturated at high temperature, and the cycle is repeated. PCR was done in all protocols in a total volume of 25 µl as follows:

x µl	Template (Genomic DNA 0.1-1 µg) or (cDNA 1 µg)
2.5 µl	10x *Taq* DNA buffer containing $MgCl_2$
0.125 µl	5 U/µl *Tag* DNA polymerase
1 µl	10 mM dNTP mix
1 µl	10 µM Sense primer
1 µl	10 µM Antisense primer
x µl	H_2O up to 25 µl

The following different PCR protocols were used in this work:

2.6.5.1. Hot start PCR

Hot start PCR reduces the generation of nonspecific products and primer artifacts. It was performed by delaying the addition of *Taq* DNA polymerase until the reaction temperature reached 94°C (when the DNA is fully denatured)

The programme was:

95°C	5 min
70°C	pause (addition of *Taq* DNA polymerase)
94°C	30 sec
47-58°C	40 sec
72°C	2 min 3 – 34 cycles
94°C	40 sec
47-58°C	40 sec
72°C	10 min
12°C	pause

2.6.5.2. Touchdown PCR (TD-PCR)

In this form of PCR the goal can be achieved by selecting a broad range of annealing temperatures, that begins above the estimated melting temperature and ends below it, thereby increasing the specificity of the PCR.

95°C	5 min
70°C	pause
94°C	30 sec
60°C	30 sec
72°C	90 sec 3 – 10 cycles
94°C	30 sec

50°C	30 sec
72°C	90 sec 6 – 20 cycles
72°C	10 min
12°C	Pause

2.6.5.3. TATA-PCR

This is a strategy to isolate the 5`prime end of a gene from genomic DNA (Guo, unpublished), by using a degenerate primer designed according to the TATA-box ((see 2.2.3.C), which is conserved in the promoter region of eukaryotic gene, in combination with a gene specific primer of a known region. The cycling conditions were as follows:

Amplification	Cycle No.	Thermal setting
First	1	98°C[3 min]
	6	95°C[30 sec], 62°C[30 sec], 72°C[2.5 min],
	1	95°C[30 sec], 30°C[40 sec], ramping to 72°C at o.2°C/sec, 72°C[2.5 min]
	6	95°C[30 sec], 60°C[30 sec], 72°C[2.5 min]
	5	95°C[30 sec], 58°C[30 sec], 72°C[2.5 min]
	10	95°C[30 sec], 56°C[30 sec], 72°C[2.5 min]
	1	72°C[5 min]
Second and Third	1	98°C[3 min]
	4	95°C[30 sec], 65°C[30 sec], 72°C[2.5 min]
	4	95°C[30 sec], 62°C[30 sec], 72°C[2.5 min]
	4	95°C[30 sec], 59°C[30 sec], 72°C[2.5 min]
	18	95°C[30 sec], 56°C[30 sec], 72°C[2.5 min]
	1	72°C[5 min]

2.6.5.4. Restriction site PCR (RS-PCR)

(Sarkar et al, 1993)

A direct method that rapidly retrieves unknown sequences adjoining a known sequence, that utilizes specially designed primers that recognize, anneal and sustain PCR, these primers are generated corresponding to any restriction enzyme(Pf1 and Pf2, see 2.2.3.B) in combination with gene specific primers, the condition used was as follows:

First amplification:

1.5 µl	10 x *Pfu* buffer
1 µl	10 µM Universal primer (Pf1 or Pf2)
6 µl	0.1-1 µg Template (Genomic DNA)

↓ 98°C 5 min
70°C pause

1 µl	10 x *Pfu* buffer
1 µl	dNTP mix (10 mM)
0.25 µl	3 U/µl *Pfu* DNA polymerase
xµl	Up to 24 µl H_2O

↓ 20°C 40 sec
68°C 5 min
95°C 40 sec 3 - 5
70°C pause

Addition of gene specific primer 1 µl (10 µM)

↓ 51-55°C 40 sec
68°C 5 min
95°C 40 sec 7 – 29
68°C 10 min
12°C pause

Second amplification:

Was performed as described under hot start PCR (see 2.6.5.1.).

2.6.5.5. Site directed mutagenesis (SDM-PCR)

This PCR technique was performed in order to make a point mutation in a DNA fragment that was inserted in a super coiled double-stranded DNA vector. Two synthetic oligounucleotide primers containing the desired mutation (in the middle of the primer sequence) were designed (see 2.2.3.F). During extension of the mutagenic primers in PCR cycles catalyzed by *Pfu* DNA polymerase, a mutated plasmid containing nicked circular strands was generated. The following conditions were used:

1.25 µl	10 mM Sense mutagenic primer
1.25 µl	10 mM Antisense mutagenic primer
x µl	0.1-1 µg Template: DNA Plasmid
5 µl	10 x*Pfu* buffer
1.25 µl	10 mM dNTP mix
0.5 µl	3 U/µl *Pfu* DNA polymerase
x µl	Up to 50 µl H_2O

The program used was:

95°C	2 min
72°C	pause
50 – 60 °C	40 sec
72°C	10 min
95°C	30 sec 3 – 25 cycles
50 - 60°C	40 sec
72°C	20 min
12°C	pause

The SDM-PCR product was treated with *DpnI* to digest the parental DNA template and to select mutation-containing synthesized DNA. The enzyme *DpnI* is specific for methylated and hemimethylated DNA. After 1 h of incubation at 37°C, 1 – 5 µl *DpnI* treated DNA was used for transformation in *DH5α* competent cells (2.6.7).

2.6.6. Cloning of PCR Products

After DNA purification from agarose gels (2.6.1.3.), a recombinant plasmid was generated by ligating the DNA containing the compatible cloning restriction sites into an appropriate vector.

2.6.6.1. Cloning in pGEM-T easy Vector (Invitrogen)

The pGEM-T easy vector was used to clone target DNA. The vector was prepared by cutting with *EcoRI* and adding a 3`-terminal thymidine to both ends. The thermostable polymerase enzyme has a terminal transferase activity which adds a single deoxyadenosine to the 3`-ends of the amplified fragments, which will greatly improve the efficiency of ligation with vector later. This terminal transferase activity is the basis of the TA- cloning strategy. The ligation reaction was performed using T4 DNA ligase at 4°C overnight, with 50 – 200 ng vector (molar ratio of vector to insert is 1:3) in a 10 µl final volume.

DNA insert	5 - 7.5 µl
10x DNA ligase buffer	1 µl
T4 DNA ligase	0.5 µl
Plasmid vector	1 - 3 µl
Water	Up to 10 µl

At the end of the reaction, 5 µl of the mixture were subjected to transformation.

2.6.7. Transformation

(Ausubel et al., 1994)

The transformation technique refers to uptake of foreign plasmid DNA by bacterial cells and uses those bacteria to produce large quantities of the plasmid. For an effective transformation of bacteria, the cells need to be made competent, which is achieved by making small holes in the bacterial cell walls by suspending them in a solution of high calcium concentration.

2.6.7.1. *E. coli* DH5αTM

This strain of *E. coli* was used for initial cloning of target DNA in pGEM T easy vector and also for plasmid maintenance; because this strain has high transformation efficiency and high plasmid yields. This type of vector contains the coding information for the carboxyl-terminal portion of *β*-galactosidase (*lac Z*), making it useful in blue/white colony selection. The following procedure was used:

- Frozen *E. coli* DH5α competent cells (50 – 100 µl aliquot) was taken from -80°C freezer and placed on ice for 5 min
- 5 µl ligated sample was incubated with *E. coli* DH5α competent cells on ice for 20 min
- Heat shock for 45 sec at 42°C, the sample was placed on ice for 5 min
- Outgrowing the cells in 250 µl SOC medium at 37°C at 225 rpm for 90 min
- Plating the cells on selective LB agar medium containing ampicillin, IPTG and X-Gal (2.2.1.2.) followed by incubation at 37°C overnight

2.6.8. Selection of positive recombinants

2.6.8.1. Blue/White colony selection

This selection was used mainly for cloning of DNA fragments into pGEM T easy vector (2.6.6.1). The principle is based on the ability of *β*-galactosidase to produce a

blue cleavage product from an artificial chromogenic substrate, 5-bromo-4-chloro-3-indolyl- *β*-D-galactoside (X-Gal) added on selective LB-plates (2.2.1.2.). *β*-Galactosidase is generated by α complementation of the amino-terminal fragment of *ß*-galactosidase encoded by the vector carrying the *Lac*Zα gene and the carboxy-terminal portion of *ß*-galactosidase encoded by the genome of the host cell. Introduction of a cloned DNA fragment into the multicloning site of the vector, which is embedded in the coding region of the *Lac*Zα gene, disrupts the amino-terminal fragment of *ß*-galactosidase, which is no longer capable of producing active *β*-galactosidase after a complementation. That allows the differentiation between the white cell colonies which contain a DNA fragment in the cloning site of the plasmid from the blue cell colonies which contain the original plasmid without interruption of LacZα gene by foreign DNA. Plasmid was isolated from white colonies and screened for the presence of insert by restriction analysis.

2.6.8.2. Restriction analysis

This step was performed to check for the presence of target DNA within the vector. Plasmid DNA was obtained from a normal spin mini preparation (2.6.1.4). Restriction enzymes are also called restriction endonucleases, which recognize a particular sequence of DNA bases and catalyze the cleavage of the double-stranded DNA making either blunt or sticky ends.

The restriction analysis was performed in a 12 µl volume as follows:

Plasmid DNA	10 µl
10 x restriction buffer	1 µl
Restriction enzyme	1 µl

The reaction was incubated at 37°C for 1 h, the sample was loaded on an agarose gel (2.2.4.1), the size of the band was visualized and confirmed under UV-light, and the plasmid with target DNA was prepared for sequencing.

2.6.8.3. Sequence analysis

The cloned DNA fragments were sequenced using both T7 and SP6 primers (2.2.3.A). Sequencing reactions were carried out using an ABI PRISM® 377 DNA sequencer (Applied Biosystems). The cloned DNA fragment to be analyzed were labeled with multiple fluorescent dyes, loaded in the system`s vertical gels, electrophoresed, laser detected and automatically analyzed by computer.

2.6.8.4. Computer-assisted sequence analysis

The BLAST (Basic Local Alignment Tool) program was used to compare nucleotide or protein sequences to sequences in the data base and to identify members of gene families. The DNA sequence samples were analyzed using the DNAStar lasergene computer program.

2.6.9 Expression of BIS Genes

The expression patterns of BIS1 and BIS2 in *P. communis (conference cultivar)* were studied as follows:

I. Total RNA was extracted (2.6.1.1.) from two different ages of infected and non-infected leaves of *P. communis*.

II. Reverse transcription was done (2.6.4.) using 5`CDS primer (2.2.3.A).

III. PCR using expression primers specific for the BIS genes (2.2.3.G).

IV. Amplification involved 30 cycles with a denaturing time of 90 sec at 94°C, an annealing time of 45 sec, and an extension time of 10 min at 72°C.

V. The samples were loaded on agarose gels (2.2.4.1) and the result was visualized and photographed under UV-light.

2.6.10. Heterologous expression of BISs

BISs (BIS1 and BIS2) were previously cloned from young leaves of *Pyrus communis* using a primer pair derived from the 5` and 3` non coding regions of *Sorbus aucuparia* BIS (Liu et al., 2007). The recombinant proteins included a small peptide, a His_6 tag. Expression vectors were constructed for the heterologous production of the proteins in *E. coli*. The BISs were over-expressed in the bacteria as hexa-histidine-tag (6xHis) fusion proteins. The amplified open-reading frames (ORFs) were ligated into an expression vector derived from the pRSET B vector series. Recombinant plasmids were introduced into *E. coli* BL21-Codon Plus (DE3)-RIL (Stratagene) for over-expression.

2.6.10.1. *E. coli* BL21 Codon Plus (DE3)-RIL (Stratagene)

This type of *E. coli* strain was used for protein expression. The strain is a λ proghage carrying the T7 RNA polymerase gene, the *lac* UV5 promoter and *lac*I gene. The *lac* UV5 promoter is inducible by IPTG for the transcription of T7 RNA polymerase. The following procedure was used:

- Frozen *E. coli* BL21 competent cells (a 50 – 100 µl aliquot) was taken from -80°C freezer and placed on ice for 5 min
- 1-5 µl plasmid sample was incubated with competent cells on ice for 20 min
- Heat shock for 20 sec at 42°C, the sample was placed on ice for 2 min
- Outgrowing of cells in 900 µl SOC medium at 37°C at 225 rpm for 60 min
- The sample was centrifuged at 5000 rpm for 5 min
- Plating the cells on selective LB agar medium containing ampicillin and cholamphenicol (2.2.1.2.) followed by incubation at 37°C overnight; on the second day the plate was stored at 4°C

2.6.10.2. Growth of Cells and Induction of Expression

One colony containing the desired gene was inoculated into 10 ml of LB medium containing 100 µg/ml ampicillin and 30 µg /ml chloramphenicol. Inoculated LB medium was incubated on a shaker (225 rpm) at 37°C over-night. About 4 ml of the overnight culture was inoculated into 100 ml of the LB medium without antibiotics. The culture was incubated on a shaker (225 rpm) at 37°C for 2-4 h until OD_{600} = 0.6–0.8. A 0.5 ml sample was immediately taken before induction (non-induced control) and was stored at –20°C until SDS-PAGE analysis. Then IPTG was added to a final concentration of 1 mM. The culture was grown at 25°C with vigorous shaking for 4 hours. A second 0.5 ml sample (induced control) was collected and was stored at –20°C until SDS-PAGE analysis. The aliquots of induced and control cultures were then analyzed for expression of the recombinant protein by SDS-PAGE followed by Coomassie blue staining or silver staining as described later. After the culture had

been treated with IPTG, the cells were harvested by centrifugation and frozen at -20°C for further use.

2.6.10.3. Preservation of bacteria containing target gene

Before induction step, when the bacterial culture had reached OD_{600} = 0.6-0.8, an aliquot of 750 µl of the bacterial culture was mixed with 250 µl autoclaved stock solution (40 ml LB medium + 60 ml glycerol), vortexed and stored in -80°C for long term storage. Recovery of the bacteria from frozen culture was by scratching with a sterile loop and streaking on the surface of an LB agar plate containing the appropriate antibiotic.

2.7. General biochemical methods

2.7.1. Extraction of expressed proteins from *E. coli* cells

Mechanical disruption of the cell membrane (sonication) is a common method for breaking the cell membrane and isolating the soluble protein. The frozen cell pellet from a 50 ml culture (2.6.10.2) was re-suspended in 3 ml of lysis buffer, pH 8 at 4°C. Sonication of the cells was carried out on ice for 5 min at 50% pulses using a Branson Sonifier B15. After centrifugation at 14000 rpm and 4ºC for 15 min, the supernatant was stored on ice until it was applied to a Ni-NTA protein purification system (QIAGEN). An aliquot of the supernatant had been taken as expressed protein control and stored at -20°C until SDS-PAGE.

2.7.2. Purification of 6xHis-Tagged Proteins

The following purification procedure was performed on a nickel-nitrilotriacetic acid “Ni-NTA” protein purification system (QIAGEN). Ni-NTA slurry was added to 6 ml of the cleared lysate (supernatant which contained either 6xHis-BIS1 or 6xHis-BIS2) as described in (2.7.1). After mixing gently by shaking (200 rpm on a rotary shaker) at 4°C for 1 h, the lysate-Ni-NTA mixture was loaded into a column, the outlet of which was capped. After removing the outlet cap, the column was washed four times with 1 ml washing buffer each (2.2.5.1). The 6xHis tagged-fusion protein was eluted using 3.5 ml elution buffer (2.2.5.1).

2.7.3. Buffer Change and Desalting of Protein Samples

The PD_{10} column (Amersham Biosciences) was equilibrated with 25 ml 50 mM Tris-HCL buffer pH 7.5 to desalt the soluble protein. Then 2.5 ml of the protein sample was loaded onto a PD_{10}column. Soluble proteins were eluted with 3.5 ml of 50 mM Tris-HCL buffer.

2.7.4. Protein quantification

2.7.4.1. Dye-Binding Assay (Bradford, 1976)

To determine the protein concentration, the Bradford method was used which is a dye-binding assay based on the differential colour change of a dye in response to various concentrations of protein. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Blue G-250 shifts from 494 to 595 nm when binding to protein occurs. The bovine serum albumin (BSA) stock solution of 1 mg/ml was diluted in order to obtain standard dilutions in range of 1 µg/ml to 10 µg/ml. In the assay, 10 µl sample was mixed with 990 µl Bradford dye solution (2.2.5.4) in a cuvette of one cm width at 595 nm in an UV/VIS spectrophotometer. Protein concentrations were calculated from a calibration curve.

2.7.5. Concentration of Proteins from Dilute Solutions

Centrifugal devices provided rapid and convenient concentration, purification, and desalting of small volumes of biological samples. Vivaspin Centrifugal Concentrators with patented vertical membrane technology were used, where protein samples were concentrated and purified by filtration using a highly selective ultra filtration membrane. This membrane was made from polyethersulphone specifically modified to minimize protein binding. The centrifugal device was selected to have a molecular weight cutoff (MWCO) 3 to 6 times less than the molecular mass of the protein to be purified. The driving force for filtration was provided by centrifugation at 3000 to 7500 g. The protein molecules larger than the MWCO of the membrane were retained in a sample reservoir, while solvent and low molecular mass proteins passed through the membrane into the filtrate reservoir.

2.7.6. Precipitation of protein by DOC and trichloroacetic acid

This method provided rapid precipitation of low protein concentrations, to enhance sensitivity for electrophoresis or protein assay. One volume of protein solution (about 20 µg) was mixed with 1/10 volume of 0.1% DOC solution (2.2.5.4). DOC forms complexes with proteins to enhance precipitation at low pH.

Then, one volume of the previous solution was mixed with 1/10 volume of 55% TCA solution (2.2.5.4). TCA is used for precipitation of DOC-protein complexes. Then the solution was mixed and incubated overnight at 4°C. The precipitated protein was centrifuged for 15 min at 4°C at 14000 rpm. The supernatant was carefully removed and the pellet (concentrated protein) was re-suspended in 10 µl 1.5 M Tris-HCl, pH 8.8.

2.7.7. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

As illustrated in 2.2.4.2., the protein bands were visualized by two methods:

a. Coomassie Blue Staining Technique:

The Coomassie blue stain was used to check the successful separation of proteins in the gel. The gel was incubated at room temperature in Coomassie blue staining solution for 60 min or overnight, followed by de-staining the gel for 60 min. Both solutions were described in (2.2.5.2.B).

b. Silver Staining Technique:

In addition, the silver stain was used to check the successful separation of proteins. The silver stain method was described by Heukeshoven and Dernick (1988). The reagents for silver stain (see 2.2.5.2.B) were prepared freshly.

The SDS gel was documented by photographing with visible light in a Multi Image Light Cabinet (Biozym) and was stored in sealed plastic bags at 4°C.

2.8. Analytical methods

2.8.1. Enzyme Assays

The standard assay (Liu et al., 2007) was carried out in a final volume of 250 µl containing 6.8 µM benzoyl-CoA as starter substrate, 17.8 µM malonyl-CoA as extender molecule, 0.1 M KH_2PO_4 buffer (pH 7.0) and 0.125 – 2 µg purified protein (BISs). The reaction was incubated at 37°C for 10 min, the reaction was stopped with 27.0 µl 50% acetic acid and the products were extracted twice with 0.25 ml ethyl acetate by vigorous mixing for 1 min and centrifugation at 13.000 rpm for 10 min. The organic phases were dried under vacuum and the residue was dissolved in 50 µl of 50% methanol for HPLC. Analysis of the enzymatic products was performed by HPLC. The detection wavelength was 257 nm (biphenyl) and 281 nm (4-hydroxycoumarin). Standard solutions of reference compounds served for quantification.

2.8.2. HPLC Instruments

Analysis of enzymatic products was performed by HPLC.

HPLC1:		
Detector	Waters ™ 996-Photodiode Array	
Auto sampler	Waters ™ 717 Plus	
Pump	Waters ™ 616	Millipore
Controller	Waters ™ 600 S	
Software	Millennium 2010 Chromatograhy Manager	

HPLC 2:		
Detector	Agilant HP 1200 series	
Auto sampler	Agilant HP 1200 series	
Pump	Agilant HP 1200 series	Agilant
Controller	Agilant HP 1200 series	
Software	Chemstation for LC 3D systems	

2.8.3. HPLC column

Column Type	Manufacturer
Hyperclone C_{18} 3 µm column (3.20 mm x150mm)	Phenomenex
Hyperclone C_{18} 5 µm column (4.60 mm x150mm)	Phenomenex

2.8.4. Mobile phases

The eluents were bidistilled water (2.2) containing 1% *ortho*-phosphoric acid pH 2.5 (A) and methanol (B) at a flow rate of 0.5 ml min^{-1} or 0.3 ml min^{-1} according to the column size. The enzymatic product was analyzed by HPLC using either a gradient of solvents A and B or isocratic elution (constant mixture of the solvents).

	Gradient	
Time [min]	Water [%]	Methanol [%]
0.00	50	50
2.00	50	50
12.00	30	70
20.00	10	90
21.00	5	95
23.00	5	95
25.00	50	50
30.00	50	50

2.8.5. Characterization of BISs

2.8.5.1. Determination of pH and temperature optima

The incubations were carried out as in (2.8.1) at pH values from 6.0 – 9.5 in 0.1 M KH_2PO_4 buffer with 0.5 µg purified protein at 37°C for 10 min incubation. Then the enzymatic products were analyzed by HPLC and the optimum pH was determined. At the optimum pH value, another series of incubations were performed at different temperatures between 20 to 50°C.

2.8.5.2. Determination of the optimum DTT concentration

The measurement of the optimum DTT concentration was done as in (2.8.1) where DTT concentrations were between 0 – 200 µM. After 10 min incubation at 37°C, the products were extracted and analyzed by HPLC.

2.8.5.3. Study of substrate specificity

At the pH and temperature optima, enzyme assays were performed using malonyl-CoA as extender substrate and a series of starter substrates:
Benzoyl-CoA, *o*-hydroxybenzoyl-CoA, *m*-hydroxybenzoyl-CoA, *p*-hydroxybenzoyl-CoA, cinnamoyl-CoA, *p*-coumaryl-CoA, butyryl-CoA, isovaleryl-CoA, acetyl-CoA, isobutyryl-CoA, hexanoyl-CoA and n-octanoyl-CoA, after incubation at 37°C for 10 min, the products were extracted and analyzed by HPLC.

2.8.5.4. Determination of K_m values

The kinetic properties were calculated from Lineweaver-Burk plots. For benzoyl-CoA the concentration was changed between 0.05 - 12 µM and malonyl-CoA was kept constant at 17.8 µM, and for malonyl-CoA the concentration was varied between 1 – 35 µM while benzoyl-CoA was constant at 6.8 µM. The extracted products were analyzed by HPLC. Three independent experiments were performed and mean values were calculated.

2.9. Disposable Plastic Ware

Commonly used disposable plastic ware was purchased in standard grade from the following companies: Sarstedt, Renner, Biozym, and Diagnostic GmbH.

2.10. Instruments

Autoclave	Vapoklav	Sterilco
Balances	Large and small scale	Sartorius
Centrifuge	Universal 32R	Hettich

	Biofuge 13	Heraeus Sepatech
	Biofuge pico	Heraeus
Clean bench	LaminAir HLB 2472	Heraeus
Electrophoresis	Mini-sub®Cell GT	BioRad
	Wide Mini-sub®Cell GT	BioRad
	Protein-Chamber	Biometra, BioRad
Heater block	Dri-block®DB 30	Techne
Incubator	HT	Infors
Microwave Oven	Optiquick	Moulinex
pH-Meter	Digital pH Meter 325	WTW (Wissenschaftlich-Technische-Werkstätten)
Photometer	Ultraspec 1000	Pharmacia Biotech
Power supply	Standard power Pack P25	Biometra
Sonifier	Branson Sonifier B15	Heinemann, Schwäbisch Gmünd, Germany
Thermocycler		Biometra
Vacuum Conc.	Genelac SF 50	Biometra
	Rotations-Vakuum-Konzentrator	CHRIST®
Water bath	Exatherm U3	Julabo

03 RESULTS

3.1. Molecular cloning of a BICS sequence from *G. lutea*

3.1.1. Isolation of total RNA

Leaves and roots of *G. lutea* served either as fresh material or frozen at - 20°C for the isolation of total RNA (2.6.1.1).

3.1.2. Reverse transcription

The synthesis of 5'-cDNA and 3'-cDNA was performed analogous to the SMART-RACE procedure (Fig. 9 and 10). In the standard reaction, 5 to 11 µl RNA were reversely transcribed into cDNA by reverse transcriptase at 42°C (2.6.4). The resulting 5'-cDNA and 3'-cDNA were used for 5'- and 3'-RACE-PCR, respectively.

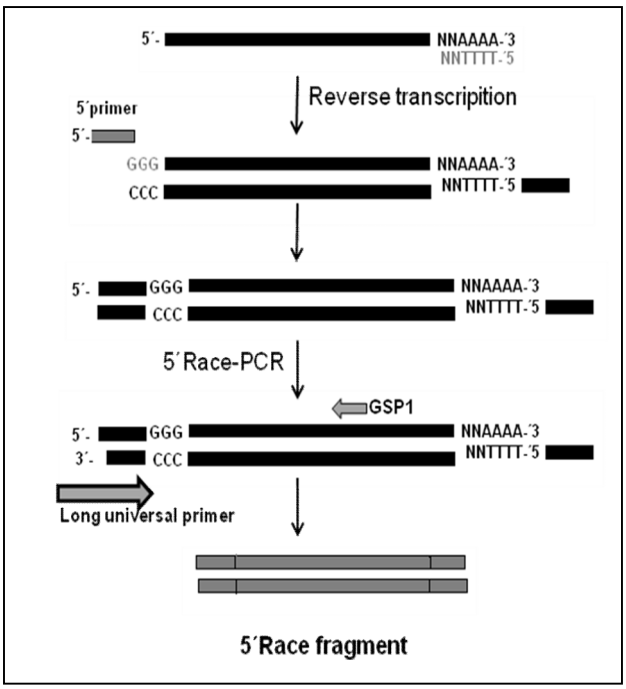


Fig. 9. Principle of 5´Race PCR

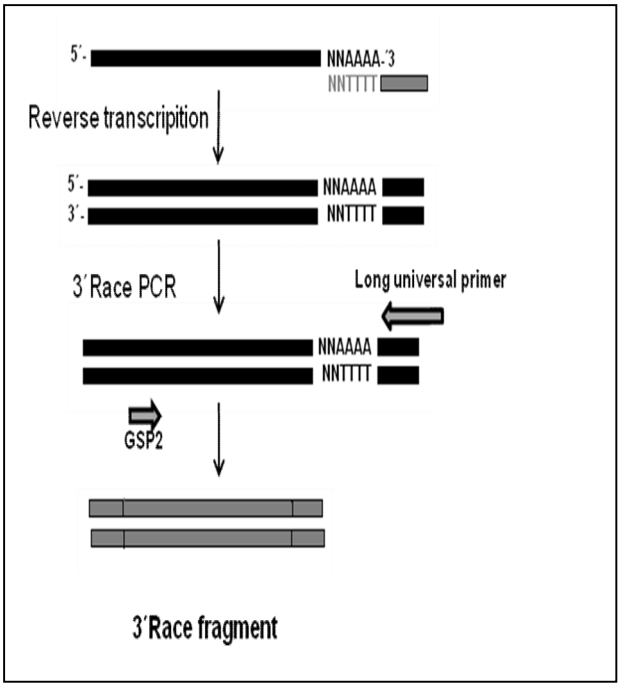


Fig. 10. Principle of 3´Race PCR

3.1.3. PCR with degenerate primers

Genomic DNA was isolated from roots of *G. lutea* (2.6.1.2) and used as template for PCR. Degenerate primers were designed according to conserved amino acid sequences in various PKSs from different plants (BPS and CHS from *H. calycinum, H. androsaemum* and *H. perforatum*). The homology of the selected type III PKSs at the amino acid level ranged from 45 – 70 % (Fig. 11). Several combinations of pairs of degenerate primers were used to search for the BICS gene. 5CHS, 5BPS, Pd5, Pd1 and Pc2 were used as forward degenerate primers and Pd3, Pd4, Pd6 and Pc1 as reverse degenerate primers. The annealing temperature chosen for PCR was a critical factor because of the degeneracy of the primers employed. To optimize the PCR, the amplification was done using a touchdown PCR protocol (2.6.5.2), in which the annealing temperature was gradually decreased (0.5°C/cycle) from 60°C to 50°C in the first 30 cycles, followed by a further 10 cycles at a constant annealing temperature of 50°C. Two core fragments were obtained and cloned into the pGEM-T easy vector (2.6.6.1) to be transformed and amplified in *E. coli* DH5α competent cells (2.6.7.1). Plasmid DNA was isolated by minipreparation (2.2.5.3), followed by restriction analysis with EcoRI (2.6.8.2) to select a clone for sequence determination.

The two core fragments were characterized as follows

First core fragment

This fragment resulted from amplification with degenerate Pd1 forward primer and Pd3 reverse primer (2.2.3.D). The amplified fragment size was 560 bp sharing 84% identity with CHS from BLAST (Basic Local Alignment Tool program; 2.6.8.4), as documented in the following.

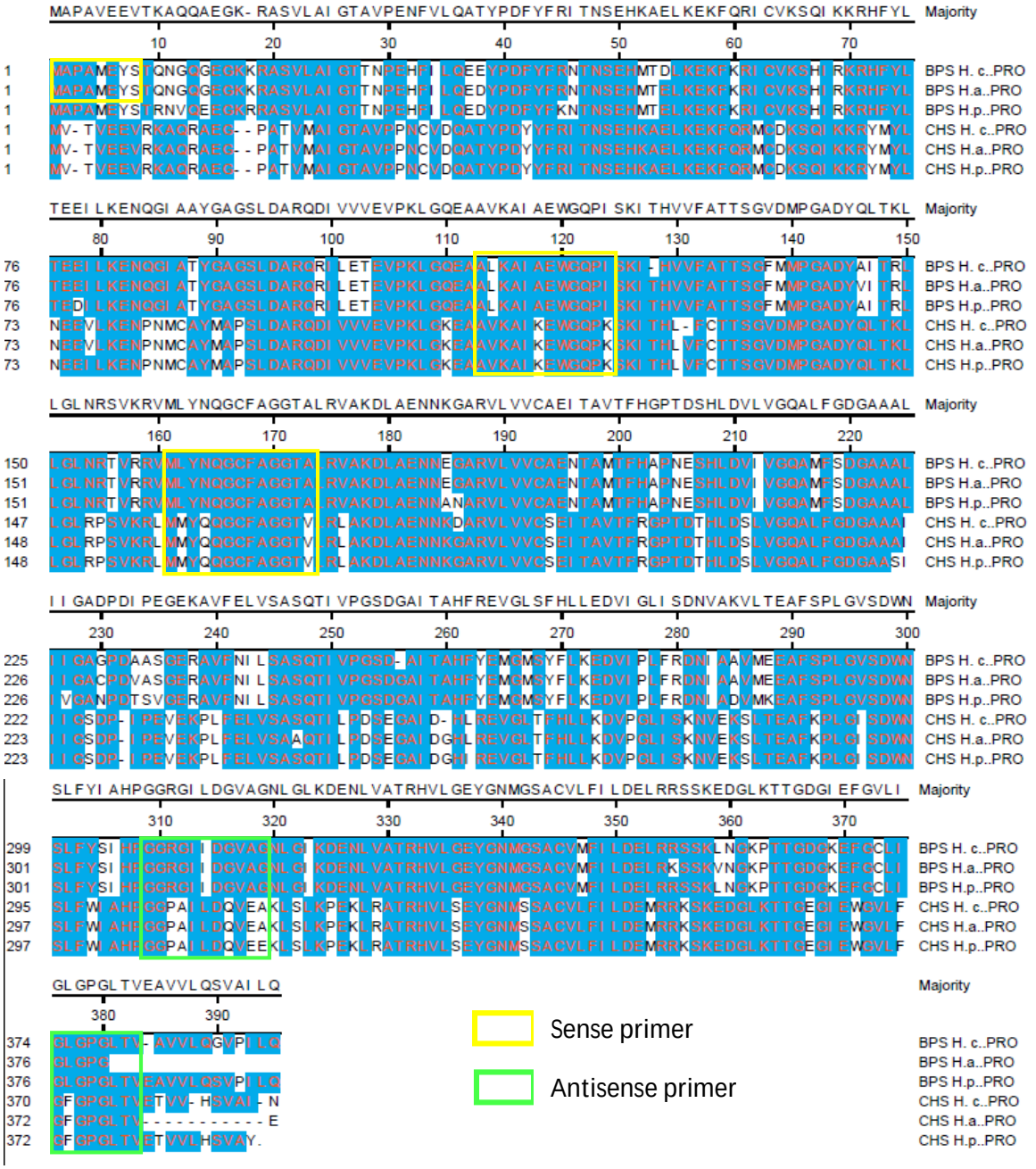


Fig. 11. Alignment of polyketide synthases from *Hypericum* species. Coloured boxes indicate the locations of degenerate primers used. BPS, benzophenone synthase; CHS, chalcone synthase; H.c., *H. calycinum*; H.a., *H. androsaemum*; H.p., *H. perforatum*

Nucleotide sequence of core fragment 1:

5´-GCGATCAAAGAATGGGGGCAGCCGATTTCTAAAATAACTCATCTAGTATTCTGCACAACAGGTGGTG
TGGACATGCCGGGTGCGGACTATCAACTCGTCAAGCTTCTTGGCCTTTGTCCGTCAGTAAAGAGATGTACCACC
AAGGTTGTTTTGCTGGGGGCACGGTCCTTAGGTTAGCCAAGGACTTAGCCGAGAACAACAAAGGTGCTCGTGT
CCTAGTTGTGTGCTCAGAGATCACTGCTATTACTTTTCGAGGCCCAAGTGAAACCCATATGGACAATCTTGTGG
GTCAAGCCATATTTGGAGATGGAGCAAGTGCAATCATAATTGGTTCAGATCCTGTCCCTGAGGTGGAGAGGCC
TTTATTTGAGCTGCTTTCTGCCACCCAGACAATTCTTCCCGATAGTCTCGGTGCGATTGAAGGACGCCTCTGTGA
AGTTGGACTAACATTCCATCTTCGTAAGGATGTTCCGAGCTTATCTCGAAGAATATTGACAAGAGTTTGAAGGA
GGCATTCGAGCCGCTAGGAATTTCTGATTGGAATTCGATTTT -3´

Amino acid sequence and data bank comparison of fragment 1:

```
>emb|CAC88858.1|  chalcone synthase [Rhododendron simsii]
Length=389

 Score =  160 bits (404),  Expect(3) = 1e-64
 Identities = 98/116 (84%), Positives = 109/116 (93%), Gaps = 0/116 (0%)
 Frame = +3

Query  132  MYHQGCFAGGTVLRLAKDLAENNKGARVLVVCSEITAITFRGPSETHMDNLVGQAIFGDG  311
            MY QGCFAGGTVLRLAKDLAENNKGARVLVVCSEITA+TFRGPS+TH+D+LVGQA+FGDG
Sbjct  159  MYQQGCFAGGTVLRLAKDLAENNKGARVLVVCSEITAVTFRGPSDTHLDSLVGQALFGDG  218

Query  312  ASAIIIGSDPVPEVERPLFELLSATQTILPDSLGAIEGRLCEVGLTFHLRKDVPSL  479
            A+AII+G+DPVPEVE+PLFEL+SA QTILPDS GAI+G L EVGLTFHL KDVP L
Sbjct  219  AAAIIVGADPVPEVEKPLFELVSAAQTILPDSDGAIDGHLREVGLTFHLLKDVPGL  274


 Score = 84.7 bits (208),  Expect(3) = 1e-64
 Identities = 40/44 (90%), Positives = 40/44 (90%), Gaps = 0/44 (0%)
 Frame = +1

Query  1    AIKEWGQPISKITHLVFCTTGGVDMPGADYQLVKLLGLCPSVKR  132
            AIKEWGQP SKITHLVFCTT GVDMPGADYQL KLLGL PSVKR
Sbjct  113  AIKEWGQPKSKITHLVFCTTSGVDMPGADYQLTKLLGLRPSVKR  156


 Score = 47.4 bits (111),  Expect(3) = 1e-64
 Identities = 21/25 (84%), Positives = 24/25 (96%), Gaps = 0/25 (0%)
 Frame = +2

Query  476  LISKNIDKSLKEAFEPLGISDWNSI  550
            LISKNI+K+L EAF+PLGISDWNSI
Sbjct  274  LISKNIEKALTEAFQPLGISDWNSI  298
```

Second core fragment:

This fragment resulted from amplification with Pd5 forward primer and Pd4 reverse primer (2.2.3.D). The amplified fragment size was 345 bp sharing 67% identity with CHS from BLAST (Basic Local Alignment Tool program; 2.6.8.4).

Nucleotide sequence of core fragment 2:

5´-CAACAAAGGTGCACGAGTTCTTATTGTTTGCTCGGAGATCATCGGACTTACTTTCCGGGGACCGAGC
GAAACAGATTTAGATGGTCTAGTCGGACAGGCCTTATTTGCAGATGGCGCTGCATCGTTGGTAGTCGGTTCAN
ACCCCATCCCAGGATTAGAAAAGCCTGTGTTTGAGATTGTTTCAGCTGCCCAAACTTTTATTCCAGAGAGTCAT
GGTTCCATTACCGGTGATCTTCGTGAGGCTGGCCTTATTGTCAAGCTAAGTAAAAATGTTCCAAAGTTTTTCGG
AGAGAATATTGATAAGTGCTTGGATGAGGCCTTTCAACCTCTGGGGATTACTGATTG- 3´

Amino acid sequence and data bank comparison of fragment 2:

```
>sp|Q9ZRS4.1|CHSY_CATRO  RecName: Full=Chalcone synthase; AltName: Full=Naringenin-chalcone
synthase
 emb|CAA10511.1|  chalcone synthase [Catharanthus roseus]
Length=389

 Score =  160 bits (404),  Expect = 6e-38
 Identities = 77/114 (67%), Positives = 93/114 (81%), Gaps = 0/114 (0%)
 Frame = +2

Query  2    NKGARVLIVCSEIIGLTFRGPSETDLDGLVGQALFADGAASLVVGSXPIPGLEKPVFEIV  181
            NKGARVL+VCSEI  +TFRGPSE+ LD LVGQALF DGAA+++VGS PIP +E+P+FE+V
Sbjct  181  NKGARVLVVCSEITAVTFRGPSESHLDSLVGQALFGDGAAAIIVGSDPIPEIERPLFELV  240

Query  182  SAAQTFIPESHGSITGDLREAGLIVKLSKNVPKFFGENIDKCLDEAFQPLGITD  343
            SAAQT +P+SHG+I G LRE GL   L K+VP    +NI K LDEAFQPLGI+D
Sbjct  241  SAAQTLLPDSHGAIDGHLREVGLTFHLLKDVPGLISKNIGKALDEAFQPLGISD  294
```

3.1.4. PCR with gene-specific primers

3.1.4.1. First core fragment

5´-GCAATTAAAGAGTGGGGGCAGCCGATTTCTAAAATAACTCATCTAGTATTCTGCACAACAGGTGGTG
TGGACATGCCGGGTGCGGACTATCAACTCGTCAAGCTTCTTGGCCTTTGTCCGTCAGTAAAGAGATGATTAGCC
AAGGTTGTTTTGCTGGGGGCACGGTCCTTAGGTTAGCCAAGGACTTAGCCGAGAACAACAAAGGTGCTCGTGT
CCTAGTTGTGTGCTCAGAGATCACTGCTATTACTTTTCGAGGCCCAAGTGAAACCCATATGGACAATCTTGTGG
GTCAAGCCATATTTGGAGATGGAGCAAGTGCAATCATAATTGGTTCAGATCCTGTCCCTGAGGTGGAGAGGCC
TTTATTTGAGCTGCTTTCTGCCACCCAGACAATTCTTCCCGATAGTCTCGGTGCGATTGAAGGACGCCTCTGTGA
AGTTGGACTAACATTCCATCTTCGTAAGGATGTTCCGAGCTTATCTCGAAGAATATTGACAAGAGTTTGAAGGA
GGCATTCGAGCCGCTAGGAATTTCTGATTGGAATTCGATTTT -3´

P3F1 sense primer — P6F1 antisense primer

P1F1 sense primer — P4F1 antisense primer

Fig. 12. Position of gene-specific primers in the first core fragment

In order to amplify a full-length BICS gene, gene-specific primers were designed (Fig. 12), these primers were combined with either PF1 and PF2 primers (2.2.3.B) in restriction site PCR (RS-PCR; 2.6.5.4) or a TATA primer (2.2.3.C) in TATA PCR (2.6.5.3). After repeated attempts, the first fragment was extended up to the 3´ end and about 291bp towards the 5´ end. The resulting sequence was as follows.

Nucleotide sequence of extended fragment 1:

5`-GATTGCAAAACAACCTTGGTGGTACATCGGAACTGCGACGCCGGAGAATTGTGTCAACCAAAGCCGA
TTACTATTTTAAGGTCACTAATAGCGAGCATATGACCGCCATTAAAGAAAAATTCAAGCGCATTTGTGATAAAT
CGATGATAAGGCAGCGATACATGTGTCTTACAGAAGAATTTNTAAAAGAAAATCCGAATGTGAGTGCATACAT
GGCTCCGTCGCTAGATGCTAGGCATGCAATTGCGATAGCTGAGGTTCCAAAGCTTGGAAAAGAACCAGCATTA
AAAGCAATTAAAGAGTGGGGGCAGCCGATTTCTAAAATAACTCATCTAGTATTCTGCACAACAGGTGGTGTGG
ACATGCCCGGGGCGGACTATCAACTCGTCAAGCTTCTTGGCCTTTGTCCGTCAGTAAAGAGATTTATGATGTAC
CACCAAGGTTGTTTTGCTGGGGGCACGGTCCTTAGGTTAGCCAAGGACTTAGCCGAGAACAACAAAGGTGCTC
GTGTCCTAGTTGTGTGCTCAGAGATCACTGCTATTACTTTTCGAGGCCCAAGTGAAACCCATATGGACAATCTT
GTGGGTCAAGCCATATTTGGAGATGGAGCAAGTGCAATCATAATTGGTTCAGATCCTGTCCCTGAGGTGGAGA
GGCCTTTATTTGAGCTGCTTTCTGCAACCCAGACAATTCTTCCCGATAGTATCGGTGCGATTGAAGGACGCCTC
TGTGAAGTTGGACTAACATTCCATCTTCGTAAGGATGTTCCGGAGCTTATCTCGAAGAATATTGACAAGAGTTT
GAAAGAGGCATTCGAGCCGCTAGGAATTTCTGATTGGAATTCGATTTTCTGGATAGCCCACCCCGGCGGGTCG
GCAATTCTAAACAAAATTGAGCAGAAATTAAGCCTGGAGCCCAAAAAACTCCGAGCTACAAGACATGTGTTGA
GCGAATATGGGAACATGTCGAGTGCGTCTGTCTTATTCATACTTGATGAGATGAGGAAAGCGTCGAGTAAAGA
TGGACATAGCACCACAGGAGAAGGGCTAGAATGGGGTGTGCTCTTCGGTTTTGGACCTGGTCTGACGGTCGA
GACGGTGGCTCTACGCAGTTTCAATATTTAGGGAGTATTCAGGACTCTATCCCTAATCGGGCTTGAATGTTTCC
GGCTTTATGTCTATATGAATTTGGTAATAAGCAATTGTGGGATCATTTTCTGAATAAGAGTTCGTTTTTTTTTTC
TAGAAACGATCTTTCTGTTATATACAAACAAGTATTGTAGGATGGGTGTTTTGTATTATAATTTGGACTGCAAA
GAATTCCCTATAGTGAGTCGTATTAC -3`

The sequence was now 1343 bp long and exhibited about 83% identity with CHS from BLAST (Basic Local Alignment Tool program; 2.6.8.4). New gene specific primers were designed (2.2.3.C) within the extended 5´ part. Only 20 amino acids were missing to get the full-length clone as concluded from comparison with PKSs in the data bank.

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Amino acid sequence and data bank comparison of extended fragment 1:

```
>dbj|BAD34456.1|  chalcone synthase [Eustoma grandiflorum]
Length=389

 Score =  598 bits (1543),  Expect(2) = 2e-170
 Identities = 297/356 (83%), Positives = 322/356 (90%), Gaps = 0/356 (0%)
 Frame = +3

Query  54    STKADYYFKVTNSEHMTAIKEKFKRICDKSMIRQRYMCLTEEFXKENPNVSAYMAPSLDA  233
             ST +DYYFK+TNSEHMT +KEKFKR+CDKSMIRQRYM LTEE+ KENPNVSAYMAPSLDA
Sbjct  34    STYSDYYFKITNSEHMTELKEKFKRMCDKSMIRQRYMHLTEEYLKENPNVSAYMAPSLDA  93

Query  234   RHAIAIAEVPKLGKEPALKAIKEWGQPISKITHLVFCTTGGVDMPGADYQLVKLLGLCPS  413
             R  I + EVPKLGK+ ALKAIKEWGQP SKITHL+FCTT GVDMPGADYQL KLLGL PS
Sbjct  94    RQDIVVMEVPKLGKDAALKAIKEWGQPKSKITHLIFCTTSGVDMPGADYQLTKLLGLRPS  153

Query  414   VKRFMMYHQGCFAGGTVLRLAKDLAENNKGARVLVVCSEITAITFRGPSETHMDNLVGQA  593
             VKR MMY QGCFAGGTVLRLAKDLAENNK ARVLVVCSEITA+TFRGP ETH+D+LVGQA
Sbjct  154   VKRTMMYQQGCFAGGTVLRLAKDLAENNKDARVLVVCSEITAVTFRGPCETHLDSLVGQA  213

Query  594   IFGDGASAIIIGSDPVPEVERPLFELLSATQTILPDSIGAIEGRLCEVGLTFHLRKDVPE  773
             +FGDGA+AIIIGSDP+P VERPLF+L+SA QTILP+S GAIEG L E GLTFHL KDVP
Sbjct  214   LFGDGAAAIIIGSDPIPGVERPLFQLVSAAQTILPESDGAIEGHLRETGLTFHLLKDVPG  273

Query  774   LISKNIDKSLKEAFEPLGISDWNSIFWIAHPGGSAILNKIEQKLSLEPKKLRATRHVLSE  953
             LISKNI KSL+EAFEPLGISDWNS+FWIAHPGG AIL+++E KL LEP+KLRATRHVLSE
Sbjct  274   LISKNIMKSLEEAFEPLGISDWNSVFWIAHPGGPAILDQVEVKLGLEPEKLRATRHVLSE  333

Query  954   YGNMSSASVLFILDEMRKASSKDGHSTTGEGLEWGVLFGFGPGLTVETVALRSFNI  1121
             YGNMSSA VLFILDEMRKAS+KDG STTGEGLEWGVLFGFGPGLTVETV L S +I
Sbjct  334   YGNMSSACVLFILDEMRKASAKDGLSTTGEGLEWGVLFGFGPGLTVETVVLHSVSI  389


 Score = 27.3 bits (59),  Expect(2) = 2e-170
 Identities = 11/13 (84%), Positives = 12/13 (92%), Gaps = 0/13 (0%)
 Frame = +2

Query  26  IGTATPENCVNQS  64
           IGTATP NCV+QS
Sbjct  22  IGTATPPNCVDQS  34
```

3.1.4.2. Second core fragment

5´-CAACAAAGGTGCACGAGTTCTTATTGTTTGCTCGGAGATCATCGGACTTACTTTCCGGGGACCGAGC
GAAACAGATTTAGATGGTCTAGTCGGACAGGCCTTATTTGCAGATGGCGCTGCATCGTTGGTAGTCGGTTCAN
ACCCCATCCCAGGATTAGAAAAGCCTGTGTTTGAGATTGTTTCAGCTGCCCAAACTTTTATTCCAGAGAGTCAT
GGTTCCATTACCGGTGATCTTCGTGAGGCTGGCCTTATTGTCAAGCTAAGTAAAAATGTTCCAAAGTTTTTCGG
AGAGAATATTGATAAGTGCTTGGATGAGGCCTTTCAACCTCTGGGGATTACTGATTG-3´

P1F2 sense primer P4F2 antisense primer
P2F2 sense primer P3F2 antisense primer

Fig. 13. Position of gene specific primers in the second core fragment

Pools of total RNA were isolated from roots of *G. lutea* (2.6.1.1) and used to synthesize cDNA by RT-PCR using SMART RACE primers (2.2.3.A). The second core fragment was extended up to the 3´ end using 3´ RACE ready cDNA in combination with sense gene-specific primer (Fig. 13) and Race long primer in hot start PCR (2.6.5.1). The 3´ RACE ready cDNA had been generated by using 3´ CDS primer and gene-specific primer in RT-PCR.

Nucleotide sequence of extended fragment 2:

The length obtained was 741 bp which shared 73% identity with CHS from BLAST (Basic Local Alignment Tool program; 2.6.8.4). About 600 bp toward the 5´ end were still missing to get the full length clone.

5´-CAACAAAGGTGCACGAGTTCTTATTGTTTGCTCGGAGATCATCGGACTTACTTTCCGGGGACCGAGC
GAAACAGATTTAGATGGTCTAGTCGGACAGGCCTTATTTGCAGATGGCGCTGCATCGTTGGTAGTCGGTTCAK
ACCCCATCCCAGGATTAGAAAAGCCTGTGTTTGAGATTGTTTCAGCTGCCCAAACTTTTATTCCAGAGAGTCAT
GGTTCCATTACCGGTGATCTTCGTGAGGCTGGCCTTATTGTCAAGCTAAGTAAAAATGTTCCAAAGTTTTTCGG
AGAGAATATTGATAAGTGCTTGGATGAGGCCTTTCAACCTCTGGGGATTACTGATTGGAATTCCATTTTCTGGA
TTGCACACCCTGGTGGGGCGTTGATTTTGGACAAAGTGGAACAAAAGTTAGGCCTACATCCTAAGAAGCTAAG
AGCTACAAGACATATATTAAGTGAGTATGGAAACATGTCAAGTGTTTGTGTGTTTTTCATACTTGATGAAGTCA
GAAAGTATTCAATCAAGAATGGATTTAGCACTACTGGAGAAGGGCTAGAATGGGGTGTGCTCTTCGGTTTGG
GTCCTGGCCTAACTGTTGAAACTGTTGTTTTACGCAGTGTAGCAATTTAAGTTACTACTGTGTTTCTTGATTCAC
AGAAWGCATGTTAGGKGTGATYSCCATCTTCTTCTTATTATTTAATTATGTGTTTGTAATGGTGATCAAATAATT
GCATGGTCCAAAAAAAAAAAAAAAAAAAAAAAAAAA-3´

Amino acid sequence and data bank comparison of extended fragment 2:

```
>sp|Q9ZRS4.1|CHSY_CATRO  RecName: Full=Chalcone synthase; AltName: Full=Naringenin-chalcone
synthase
 emb|CAA10511.1|  chalcone synthase [Catharanthus roseus]
Length=389

 Score =  318 bits (814),  Expect = 5e-85
 Identities = 153/209 (73%), Positives = 177/209 (84%), Gaps = 0/209 (0%)
 Frame = +2

Query  2    NKGARVLIVCSEIIGLTFRGPSETDLDGLVGQALFADGAASLVVGSXPIPGLEKPVFEIV  181
            NKGARVL+VCSEI  +TFRGPSE+ LD LVGQALF DGAA+++VGS PIP +E+P+FE+V
Sbjct  181  NKGARVLVVCSEITAVTFRGPSESHLDSLVGQALFGDGAAAIIVGSDPIPEIERPLFELV  240

Query  182  SAAQTFIPESHGSITGDLREAGLIVKLSKNVPKFFGENIDKCLDEAFQPLGITDWNSIFW  361
            SAAQT +P+SHG+I G LRE GL   L K+VP    +NI K LDEAFQPLGI+DWNSIFW
Sbjct  241  SAAQTLLPDSHGAIDGHLREVGLTFHLLKDVPGLISKNIGKALDEAFQPLGISDWNSIFW  300

Query  362  IAHPGGALILDKVEQKLGLHPKKLRATRHILSEYGNMSSVCVFFILDEVRKYSIKNGFST  541
            IAHPGG  ILD+VE+KLGL P+KLRATRH+LSEYGNMSS CV FILDE+RK S ++G ST
Sbjct  301  IAHPGGPAILDQVEEKLGLKPEKLRATRHVLSEYGNMSSACVLFILDEMRKASARDGLST  360

Query  542  TGEGLEWGVLFGLGPGLTVETVVLRSVAI  628
            TGEGLEWGVLFG GPGLTVETVVL SV +
Sbjct  361  TGEGLEWGVLFGFGPGLTVETVVLHSVNV  389
```

All further trials of 5’ cloning failed. Thus, neither clone 1 nor clone 2 could be obtained as full-length sequences within the scope of the present work. Fig. 14 schematically displays the present state of the cloning efforts.

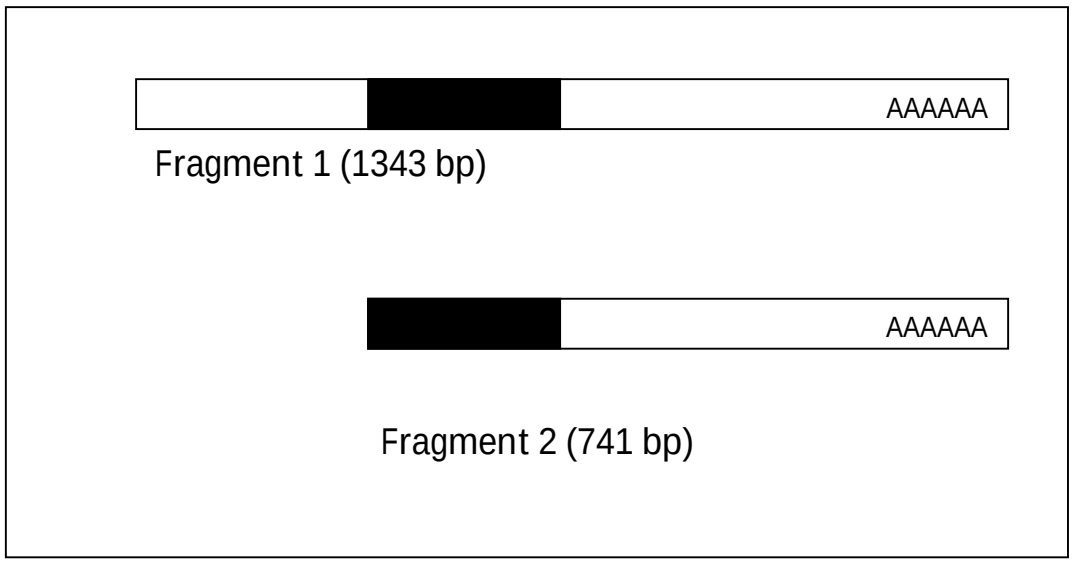


Fig. 14. Schematic representation of the two *G. lutea* clones. The core fragments initially obtained with degenerate primers are indicated by black boxes

3.2. Characterization of BISs from *P. communis*

3.2.1. Heterologous expression of BISs

This work started with a cDNA library complementary to mRNA from young, non-infected leaves of *P. communis*, which was kindly provided by Prof. Dr. T. Fischer (Biochemie und Pflanzenphysiologie, Department Biologie I, Biozentrum-Botanik, Ludwig-Maximilians-Universität München, Germany).

Two BIS cDNAs were cloned using a primer pair derived from the 5‘ and 3‘ non-coding regions of *S. aucuparia* BIS (Liu et al, 2007). The pear BISs share 98.2% amino acid sequence identity with each other and 96.2% with BIS from *S. aucuparia* (Fig. 15). They were functionally expressed in *E. coli* as 6xHis-tagged proteins (2.6.10.1).

3.2.2. Protein purification

The Ni-NTA system was used for the purification of the 6xHis-tagged BISs. BIS1 was purified from a small-scale culture because of the protein’s instability. The soluble fraction (6 ml) from *E. coli* cells (100 ml) containing 6xHis-BIS1 was applied to the affinity matrix and 6xHis-BIS1 was eluted with elution buffer (2.5 ml). The yield of fusion protein was 0.5 mg, as measured by the Bradford assay (2.7.4.1).

The 6xHis-BIS2 protein was purified using the same procedure as above. The yield of the eluted fusion protein was 1.5 mg.

3.2.3. Gel electrophoresis (SDS-PAGE)

The over-expression of the BIS recombinant proteins was confirmed by means of SDS-PAGE analysis (2.2.4.2). The following fractions were analyzed: crude bacterial protein extracts before and after induction, soluble and insoluble portions of the protein extract after induction, and 5 to 20 µg of the affinity-purified proteins (Fig. 16, 17). Protein bands were stained with Coomassie brilliant blue. Both BISs have subunit molecular masses of about 43 kDa.

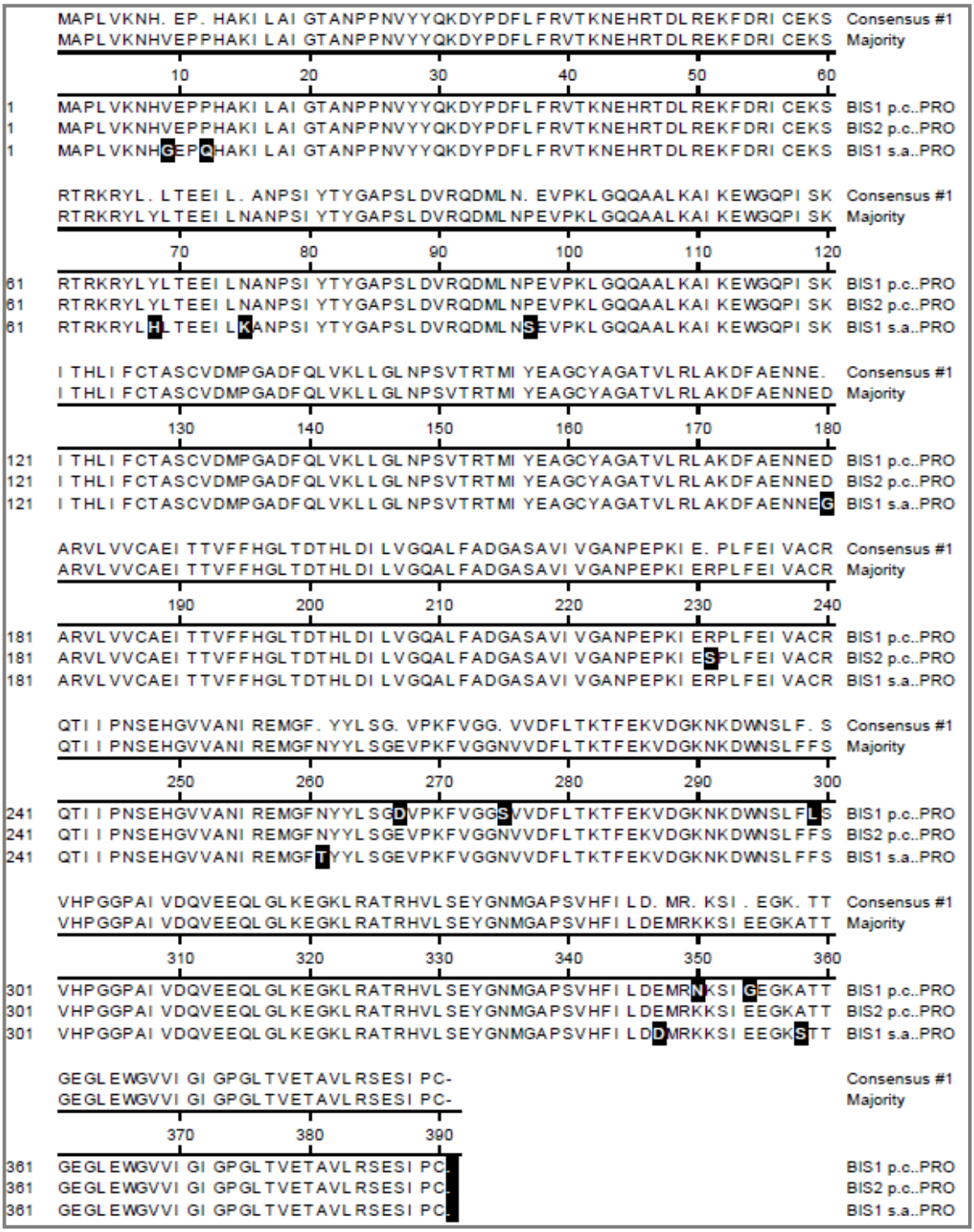


Fig. 15. Aligment of the amino acids sequences of BIS1 and BIS2 from *P. communis* (P.c.) and BIS from *S. aucuparia* (S.a.). Divergent residues are highlighted.

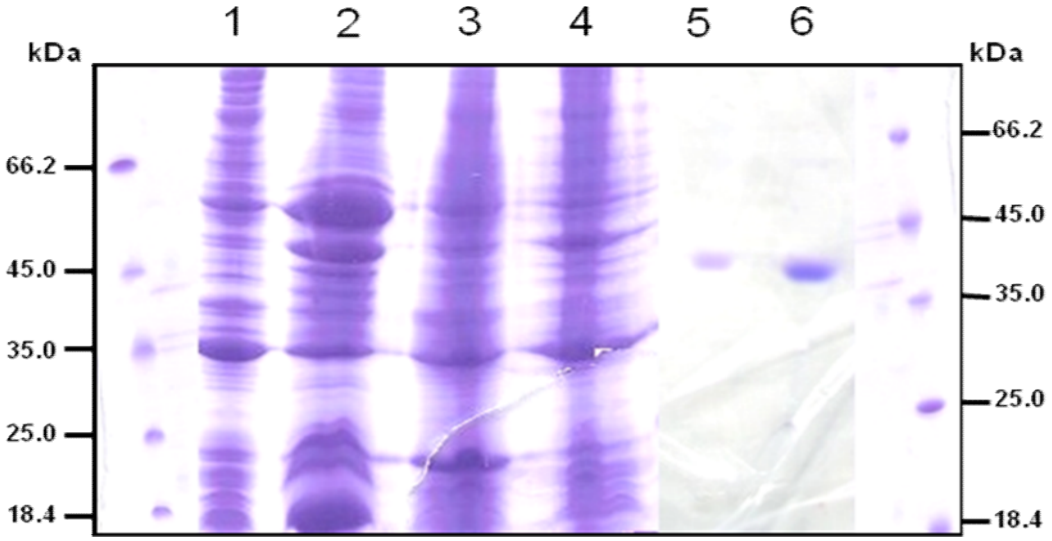


Fig. 16. SDS-PAGE (12%) of fractions containing BIS1 protein after the following purification steps, 1. pre-induction, 2. post-induction, 3. insoluble protein, 4. soluble protein, 5. 5 µg purified protein, 6. 10 µg purified protein .

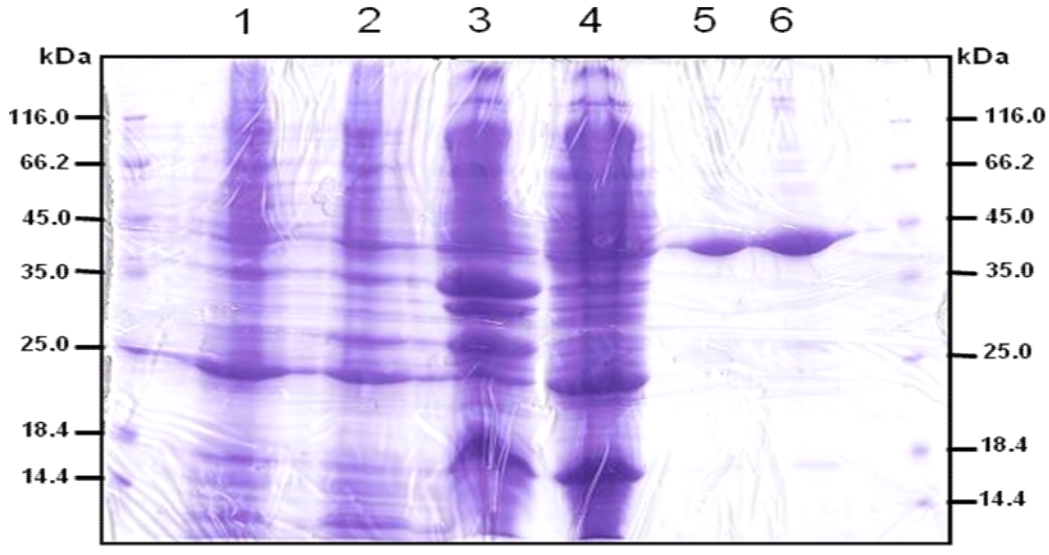


Fig. 17. SDS-PAGE (12%) of fractions containing BIS2 protein after the following purification steps, 1. pre-induction, 2. post-induction, 3. insoluble protein, 4. soluble protein, 5. 10 µg purified protein, 6. 20 µg purified protein.

3.2.4. Test for enzymatic activity

Previously conducted experiments with yeast-extract-treated *S. aucuparia* cell cultures showed the formation of aucuparin (Liu et al, 2004) and moreover a cDNA encoding the involved biphenyl synthase was cloned (Liu et al, 2007). This BIS prefers as a starter substrate benzoyl-CoA that undergoes iterative condensation with three molecules of malonyl-CoA to give 3,5-dihydroxybiphenyl via intramolecular aldol condensation. Therefore the purified pear proteins were incubated with benzoyl-CoA and malonyl-CoA. Subsequent HPLC analysis revealed formation of a product which co-chromatographed with an authentic sample of the reference compound, 3,5-dihydroxybiphenyl (Fig. 18). Incubation with heat-denatured protein showed no activity in the HPLC chromatogram, indicating the enzymatic origin of the product. These observations were true for both BIS1 and BIS2. The identity of the enzymatic product was also shown by UV-spectroscopy (Fig. 18).

3.2.5. pH optimum

The activity of the BISs was determined between pH 6.0 and 9.5 in the standard assay. The dependence of enzyme activity on pH is shown in Fig. 19, where the pH optimum was 7.0 for BIS1 and 7.0 – 7.5 for BIS2. The data shown are mean values of three experiments. Consequently, all subsequent tests were performed at pH 7.0 – 7.5 in potassium phosphate buffer.

3.2.6. Temperature optimum

Standard assays were performed at 5° intervals from 20 to 50°C and average values of three experiments were calculated (Fig. 20). The temperature optimum of BIS1 was at 30°C, that of BIS2 at 20 - 25°C. The activity of BIS2 dramatically decreased from 30°C upwards.

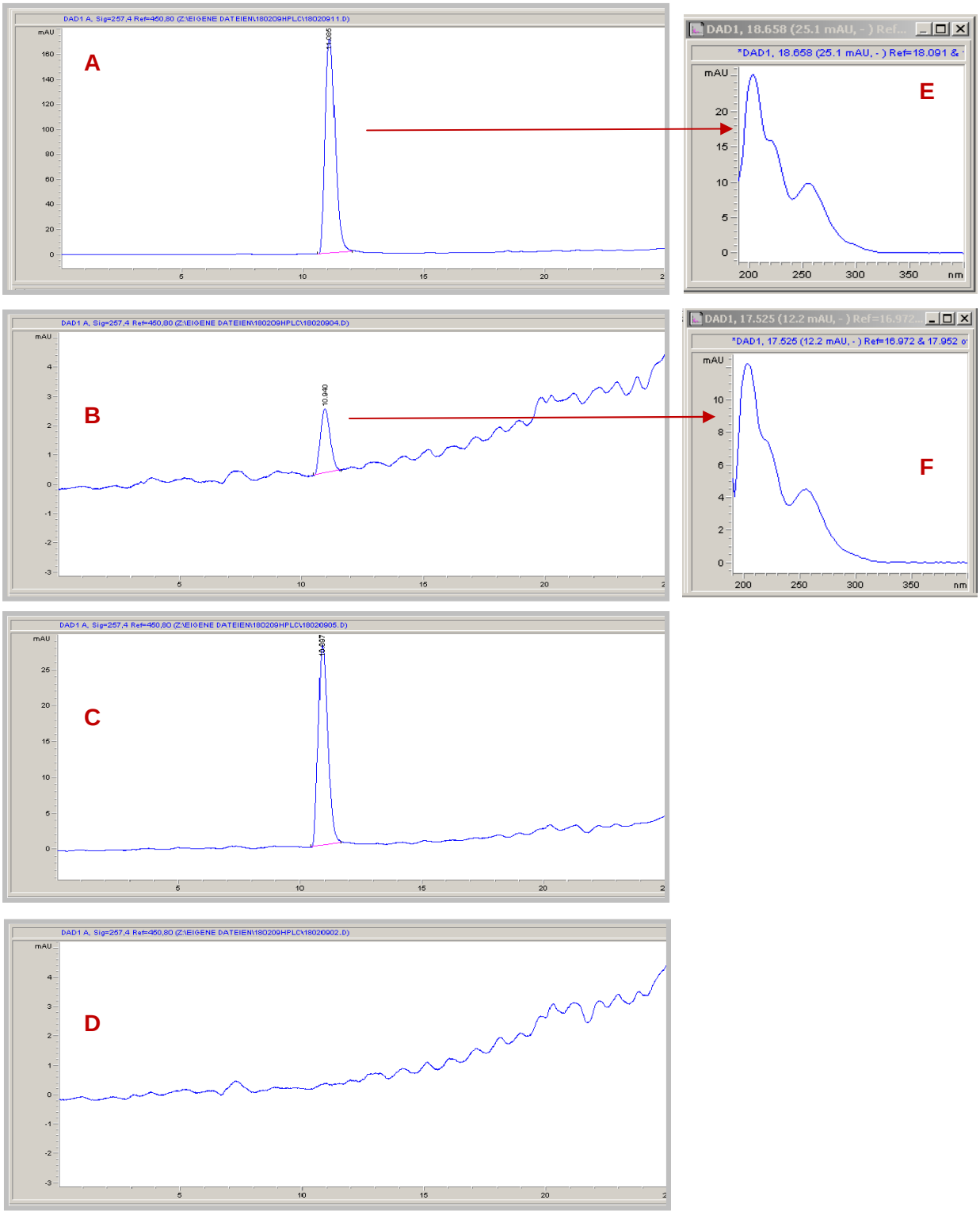


Fig. 18. 18. HPLC analysis of BIS1 assays with benzoyl-CoA, A) authentic 3,5-dihydroxybiphenyl, B) standard incubation, C) co - chromatography, D) incubation with denatured protein, E) uv-spectrun of reference , F) uv-spectrum of product in standard incubation, detection at 257 nm

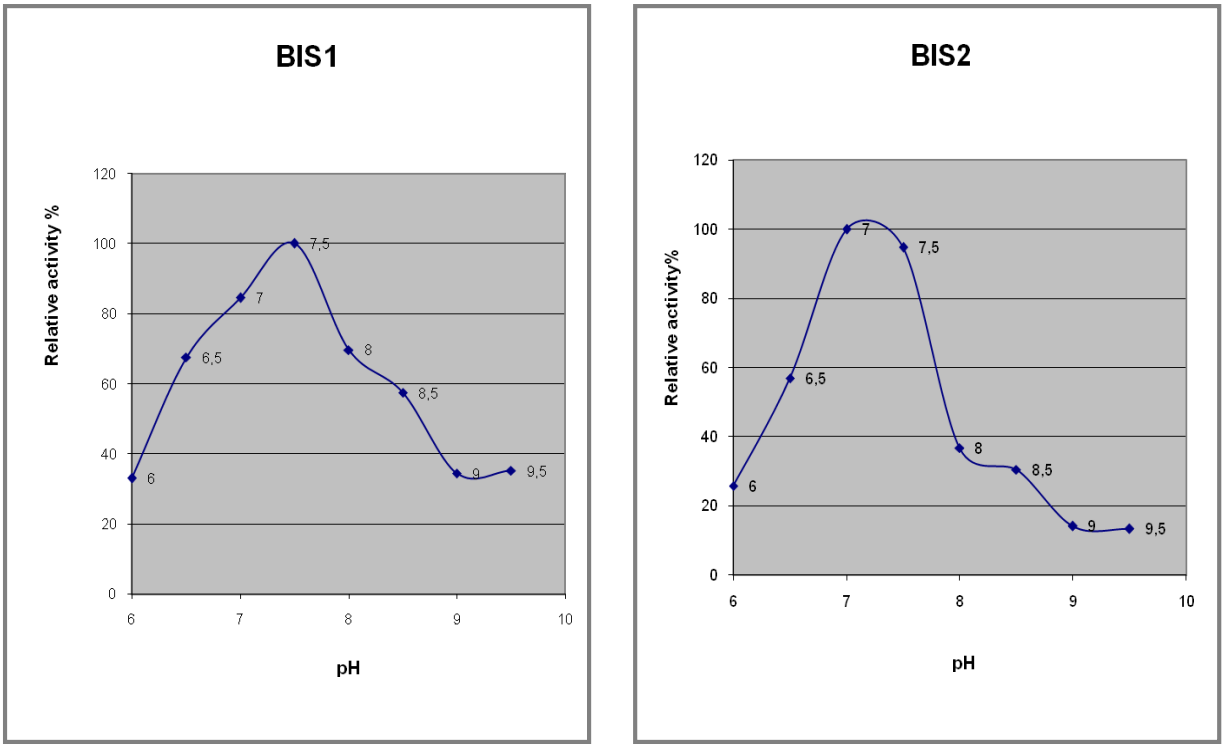


Fig. 19. pH optimum of recombinant BIS1 and BIS2

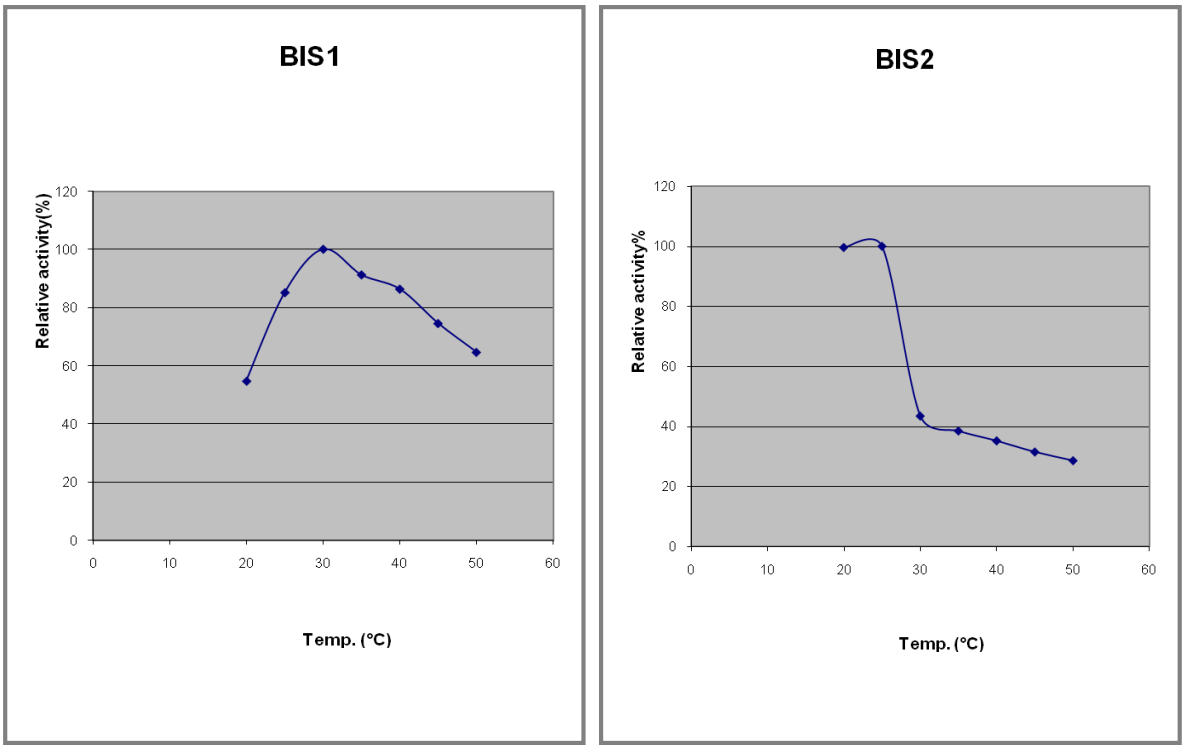


Fig. 20. Temperature optimum of recombinant BIS1 and BIS2

3.2.7. DTT dependency

DTT (dithiothreitol) is a reducing substance which stabilizes free thiol groups of amino acids. As a result of thermal stress during the incubation, the functional groups can form disulfide bonds, which influence the conformation and thus the activity. Furthermore, thiol groups are no longer available as catalytic groups. Incubations were performed in the presence of 0, 5, 10, 20, 50, 100, 150, and 200 µm DTT. Three independent experiments were performed. In contrast to BIS2 that was not appreciably stimulated, the activity of BIS1 was increased by addition of DTT (Fig. 21). However DTT significantly enhanced the formation of a yet unidentified by-product, even at a 5 mM concentration. Therefore, it was omitted in subsequent incubations.

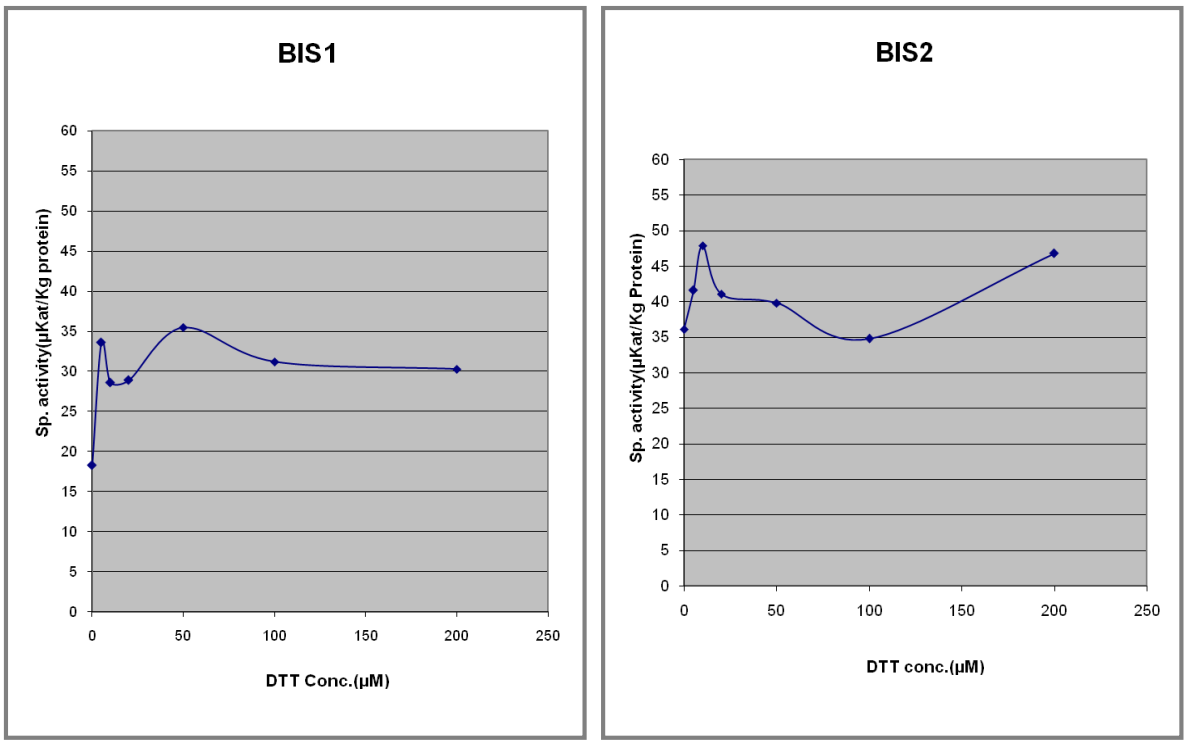


Fig. 21. Effect of DTT on the activity of BIS1 and BIS2

3.2.8. Linearity with protein amount and incubation time

The BIS1 and BIS2 reactions were approx. linear with the protein amount up to 4 µg in the standard assay (Table 2) and with the incubation time up to 30 min (Fig. 22). Product amounts were determined by HPLC using a standard solution of the authentic reference compound.

Table 2. Effect of the protein amount on the product yield

BIS1		BIS2	
Amount of protein (µg)	Amount of product (ng)	Amount of protein (µg)	Amount of product (ng)
0.125	1.43	0.125	2.58
0.250	1.49	0.250	6.26
0.500	3.21	0.500	8.49
1	6.28	1	10.18
2	10.18	2	21.60
4	22.01	4	44.72
8	28.31	8	45.21
16	25.51	16	40.11

3.2.9. Substrate specificity

Under the above-described, optimized conditions, enzyme assays were done to test the ability of the BISs to use different starter substrates. The products formed were examined by HPLC. The dectection wavelengths were 257 nm (3,5-dihydroxybiphenyl), 281 nm (4-hydroxycoumarin), and 319 nm (pyrone side products). The BISs were incubated in the presence of malonyl-CoA with benzoyl-CoA, *ortho*-, *meta*- and *para*-hydroxybenzoyl-CoA, cinnamoyl-CoA, *p*-coumaryl-CoA, butyryl-CoA, isovaleryl-CoA, acetyl-CoA, isobutyryl-CoA, hexanoyl-CoA and *n*-octanoyl-CoA (2.8.5.3). Both recombinant enzymes preferred benzoyl-CoA as a

starter substrate and formed 3,5-dihydroxybiphenyl (Table 3). No side-products were detectable. The enzymes were also active, with salicoyl-CoA, however, the enzymatic product was not the corresponding biphenyl, but 4-hydroxycoumarin, as recently observed with *S. aucuparia* BISs (Liu et al., 2010). The identity of 4-hydroxycoumarin was established by co-chromatography and UV-spectroscopy (Fig. 23). Beside 4-hydroxycoumarin, by-products were not detectable. 3,5-Dihydroxybiphenyl and 4-hydroxycoumarin were quantified using standard solutions of the respective authentic reference compounds. In contrast to *S. aucuparia* BISs (Liu et al., 2010), the pear enzymes were inactive with 3-hydroxybenzoyl-CoA. Similarly, all the other substrates used were accepted by neither BIS1 nor BIS2 from pear.

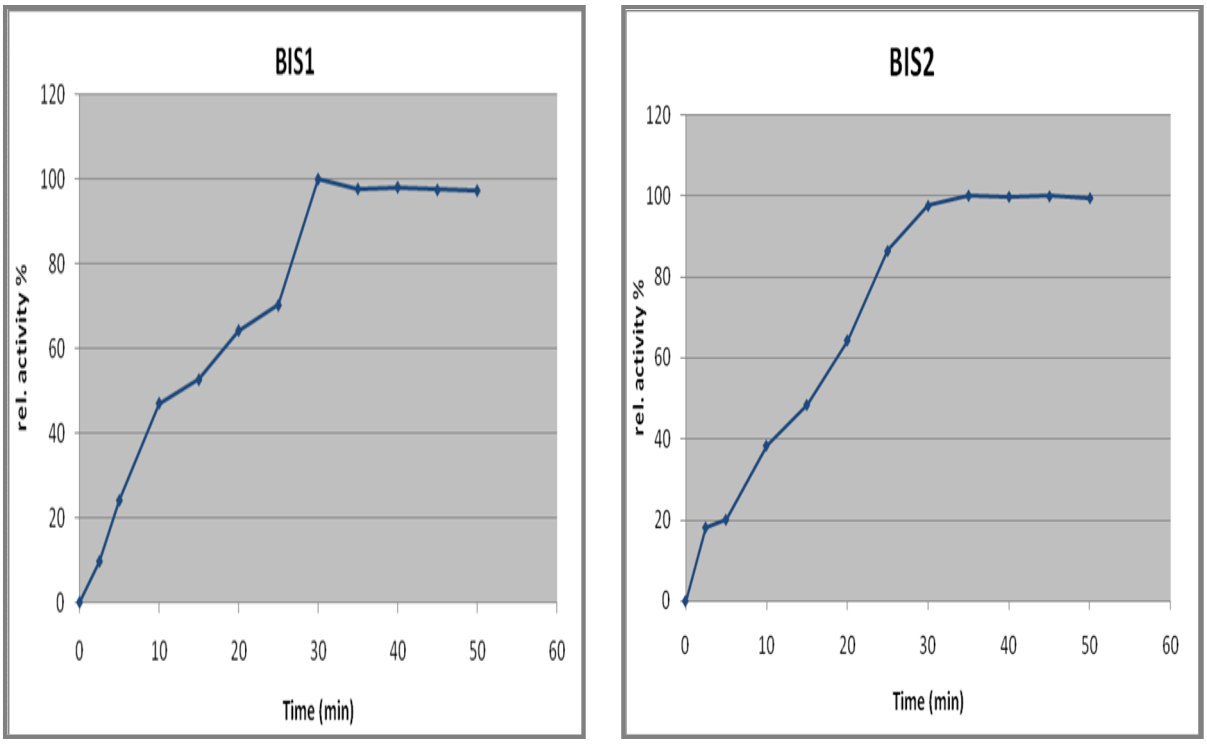


Fig. 22. Time course of product formation in BIS1 and BIS2 assays

3.2.10. Determination of kinetic parameters

Finally important for the characterization of BIS1 and BIS2 was the determination of the two kinetic constants, substrate affinity (K_m) and turnover number (K_{cat}).

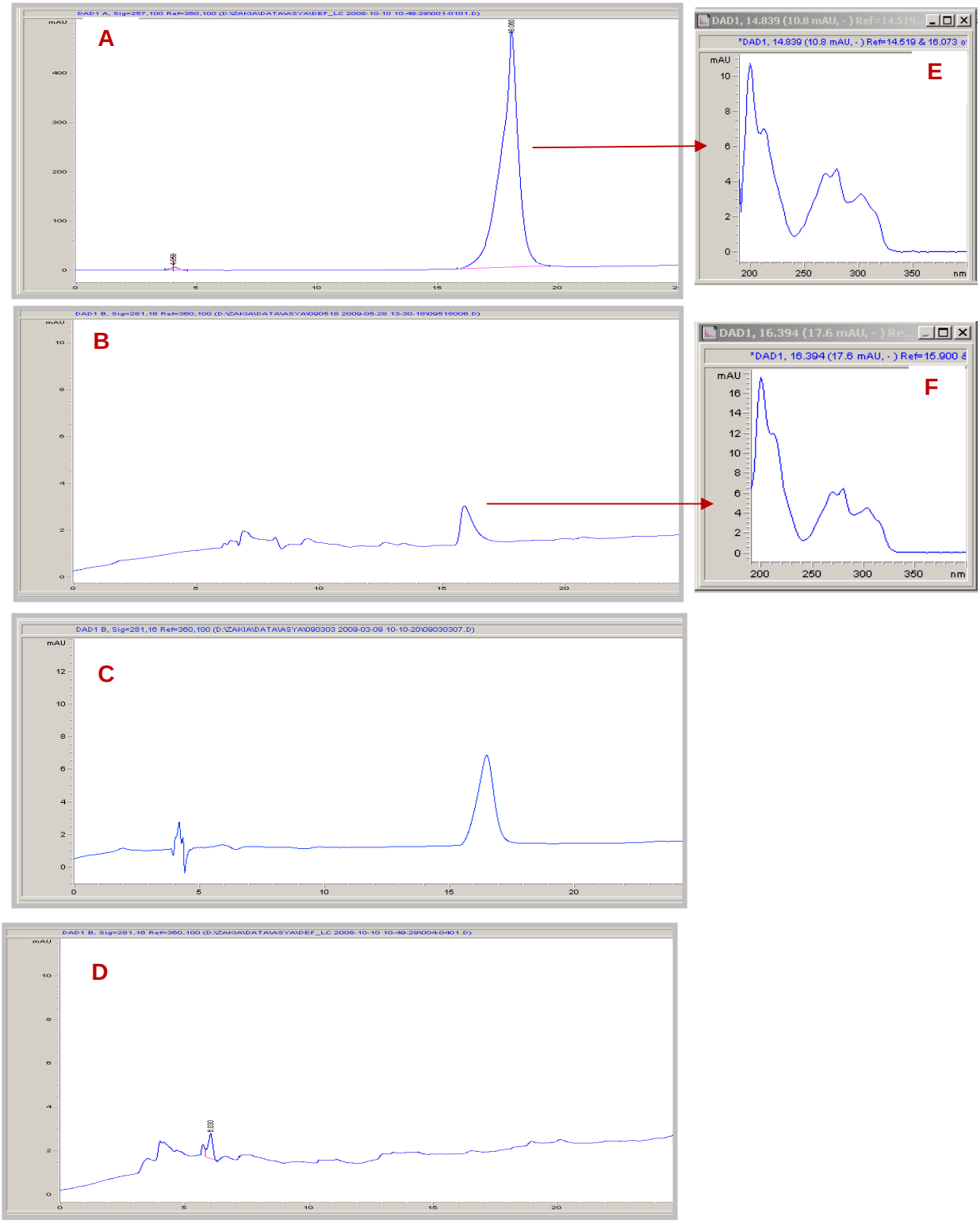


Fig. 23. HPLC analysis of BIS1 assays with salicoyl-CoA, A) authentic 4-hydroycoumarin, B) standard incubation, C) co - chromatography, D) incubation with denatured protein, E) uv-spectrun of reference , F) uv-spectrum of product in standard incubation, detection at 281 nm

The substrate affinity (Michaelis-constant) describes the dependence of the enzyme activity on the substrate concentration and is determined at Vmax/2. The K_m value was measured by linear regression of 1/v versus 1/[s], known as Lineweaver-Burk plot, which was carried out in three independent series of experiments, each containing 2.0 µg protein (2.8.1). The incubation period was limited to 10.0 min. The substrate concentrations varied for benzoyl-CoA from 0.05 to 12 µM, while malonyl-CoA was kept constant at 17.8 µM and for malonyl-CoA from 1 – 35 µM, while benzoyl-CoA was constant at 6.8 µM. The results are documented in Figs. 24-31 and summarized in Table 4.

Table 3. Substrate specificities of BIS1 and BIS2

Starter substrate	Product (% of maximum each)	
	BIS1	BIS2
benzoyl-CoA	100	100
ortho-hydroxybenzoyl-CoA	14.6[a]	23.1[a]
meta-hydroxybenzoyl-CoA	0	0
para-hydroxybenzoyl-CoA	0	0
cinnamoyl-CoA	0	0
para-coumaroyl-CoA	0	0
butyryl-CoA	0	0
isovaleryl-CoA	0	0
acetyl-CoA	0	0
isobutyryl-CoA	0	0
hexanoyl-CoA	0	0
n-octanoyl-CoA	0	0

a = formation of 4-hydroxycoumarin instead of biphenyl.

Kinetic data for BIS1

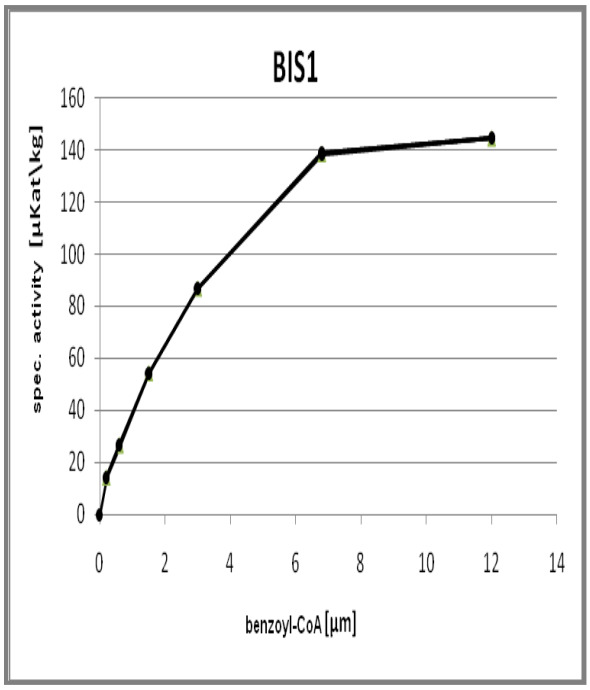


Fig. 24. Dependence of BIS1 activit on the benzoyl-CoA concentration

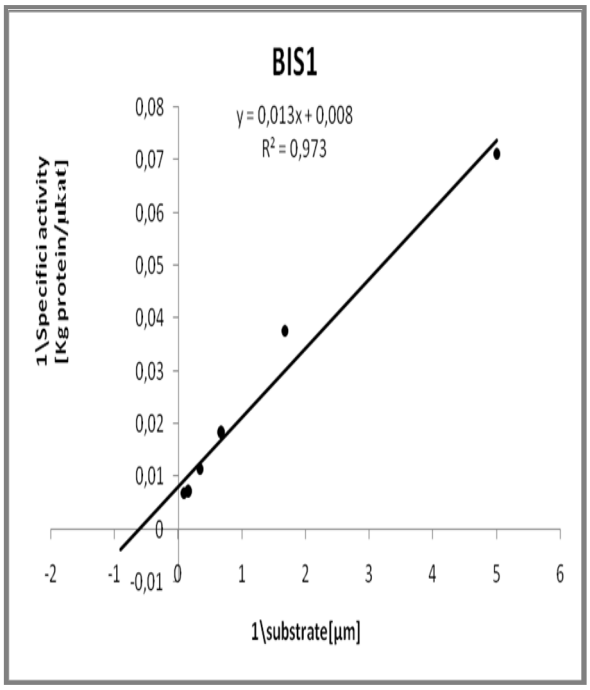


Fig. 25. Determination of the K_m value for benzoyl-CoA from a Lineweaver-Burk plot

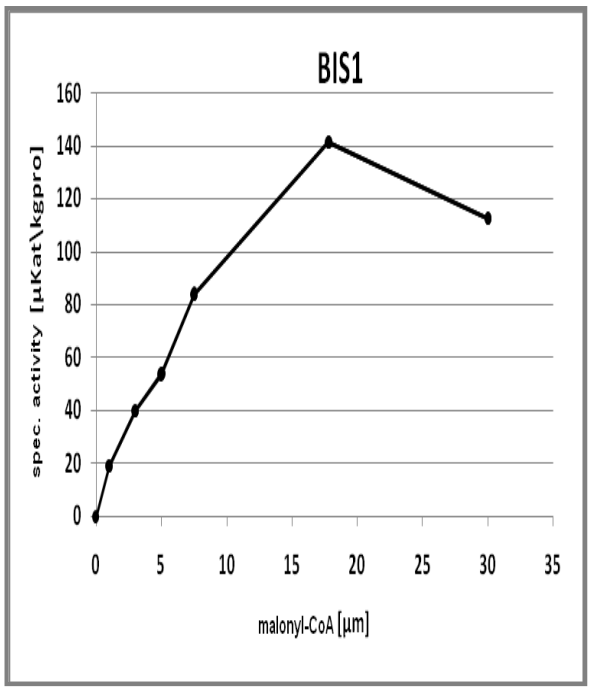


Fig. 26. Dependence of BIS1 activity on the malonyl-CoA concentration

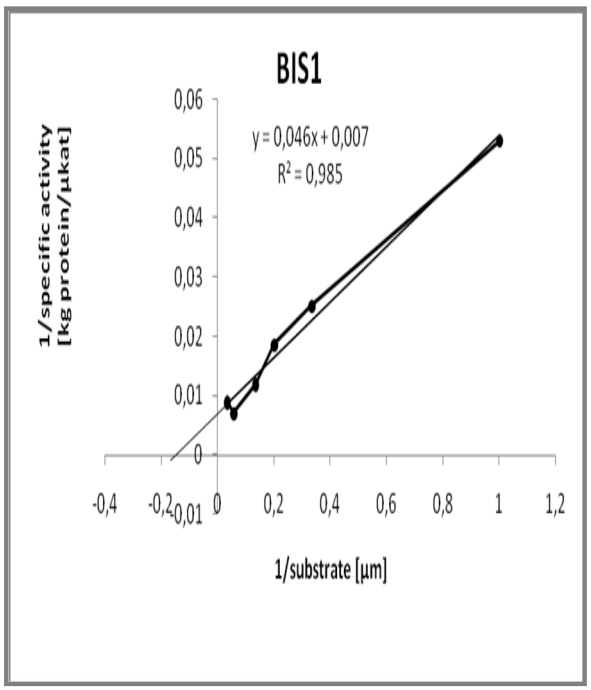


Fig. 27. Determination of the K_m value for malonyl-CoA from a Lineweaver-Burk plot

Kinetic date for BIS2

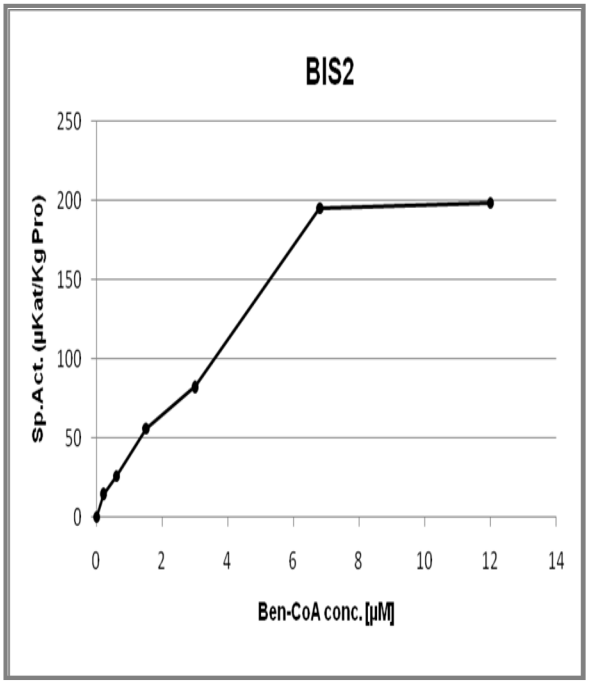


Fig. 28. Dependence of BIS2 activity on the benzoyl-CoA concentration

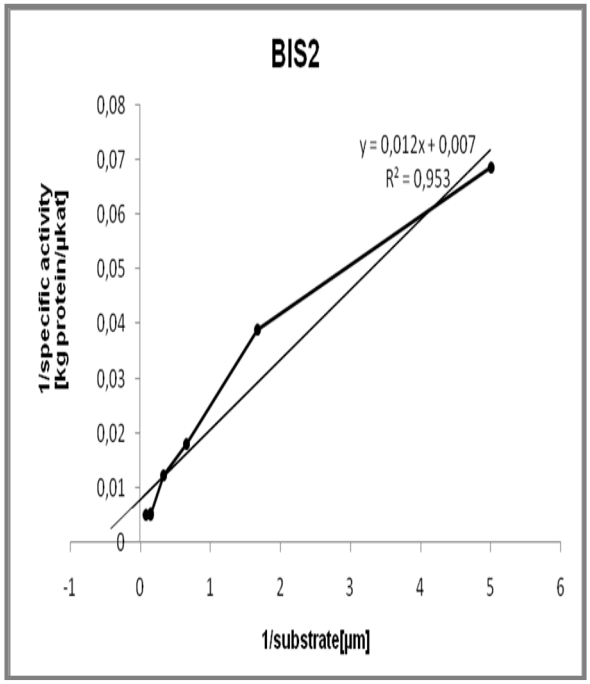


Fig. 29. Detemination of the K_m value for benzoyl-CoA from a Lineweaver-Burk plot

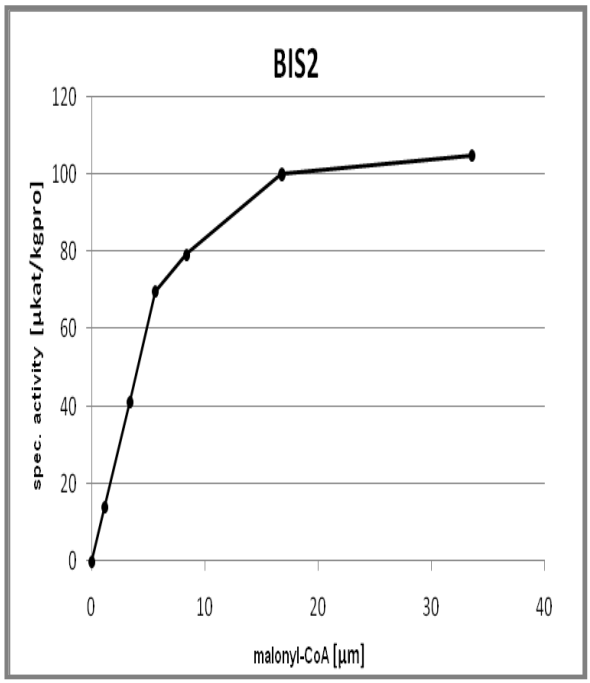


Fig. 30. Dependence of BIS2 activity on the malonyl-CoA concentration

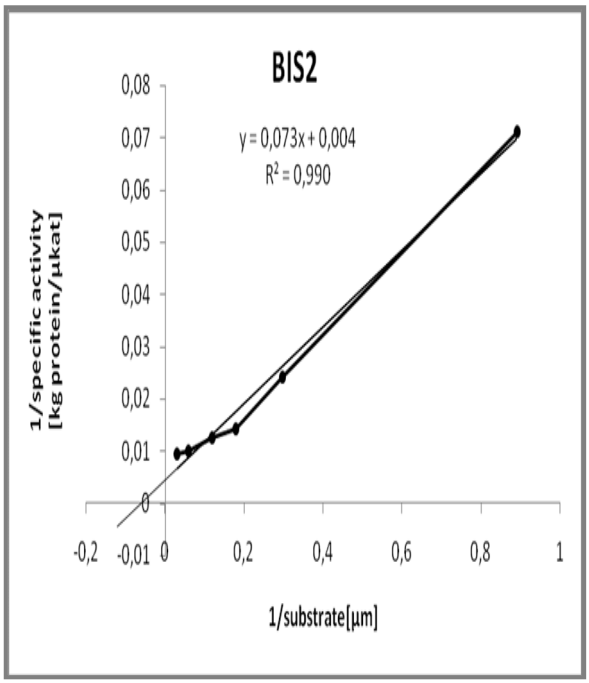


Fig. 31. Detemination of the K_m value for malonyl-CoA from a Lineweaver-Burk plot

Table 4 lists the apparent kinetic parameters. Besides the K_m values, the K_{cat} and K_{cat}/K_m values were determined on the basis of the Lineweaver-Burk plots at saturating concentrations of both substrates and under optimum conditions.

Table 4. Steady-state kinetic parameters of BIS1 and BIS2 from *P. communis*

Substrate	Parameter	BIS1	BIS2
Benzoyl-CoA	K_m(µM)	1.6	1.7
	K_{cat}(min^{-1})	0.65	0.74
	K_{cat}/K_m ($M^{-1}s^{-1}$)	6771	7255
Malonyl-CoA	K_m(µM)	6.5	17.1
	K_{cat}(min^{-1})	0.74	1.19
	K_{cat}/K_m ($M^{-1}s^{-1}$)	1897	1159

3.3. Expression of BIS1 and BIS2 genes

A growing pear plant (*P. communis*) was inoculated with fire blight by cutting the two youngest leaves using a pair of scissors dipped in a suspension of the fire blight bacterium *Erwinia amylovora* (10^9 bacteria/ml). Two and six days later, total RNA was isolated from the first leaves under the inoculation site. RT-PCR with gene-specific primers for BIS1 and BIS2 was carried out and the products were separated on an agarose gel (2.6.9). Expression of the BIS1 gene was strongly induced by

inoculation (Fig. 32). After 2 and 6 days of infection, the expected fragment size of 390 bp was detected. This band was weak in control RNA. In contrast, BIS2 expression was not induced by *E. amylovora* infection. Similar data were obtained for both the 2 cm and 6 cm leaves.

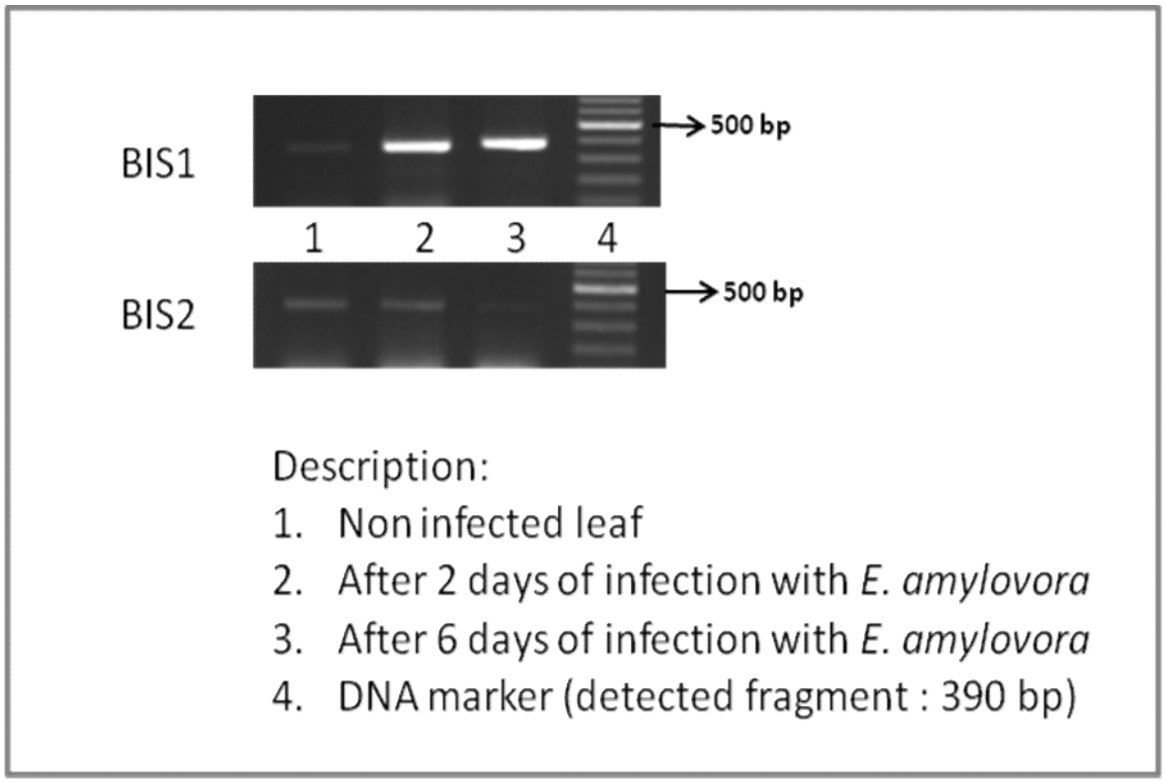


Fig. 32. Expression of BIS1 and BIS2 genes in *P. pyrifolia* leaves after inoculation with *E. amylovora*

3.4. Site-directed mutagenesis of BIS1

The high degree of amino acid sequence identity between BIS1 and BIS2 (98.2%) provoked the idea of the mutational exchange of the altered amino acids. Only six residues differ between the BIS1 and BIS2 sequences at the positions 231, 267, 275, 299, 350, 354 (Fig. 33). These residues were altered in BIS1 by site-directed mutagenesis. The resulting mutations are identified in the form that the first letter indicates the original amino acid at the corresponding position in the wild-type BIS1protein and the last letter indicates the newly introduced amino acid.

Under the PCR conditions described in 2.6.5.5, using BIS1 mutation primers, the resulting mutated plasmids were subjected to DpnI-digestion to remove the unmutated PCR template. Each PCR-mutated plasmid was transformed in *E. coli* BL21 for protein over-expression. The Ni-NTA system was used for the purification of the 6xHis-tagged BIS1 mutants (2.7.2), which were then subjected to BIS assays to characterize their catalytic activity and substrate specificity (2.8.1).

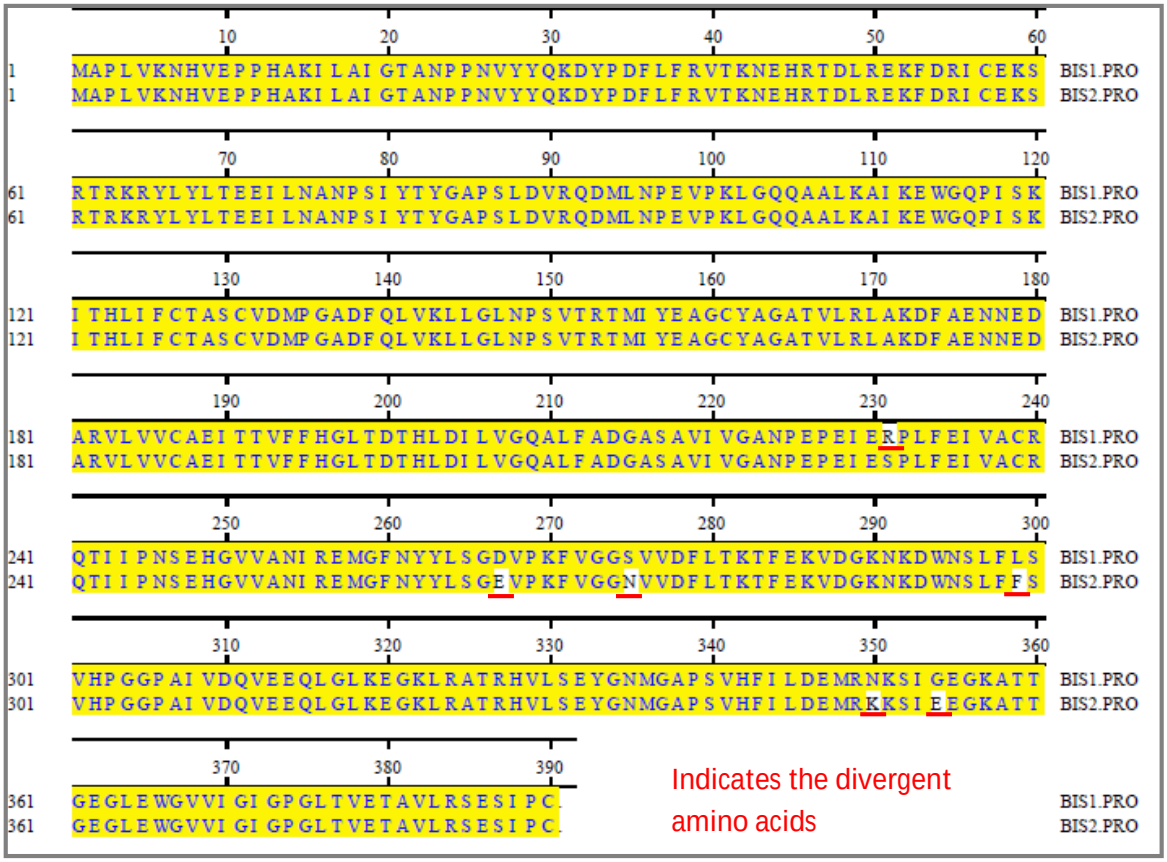


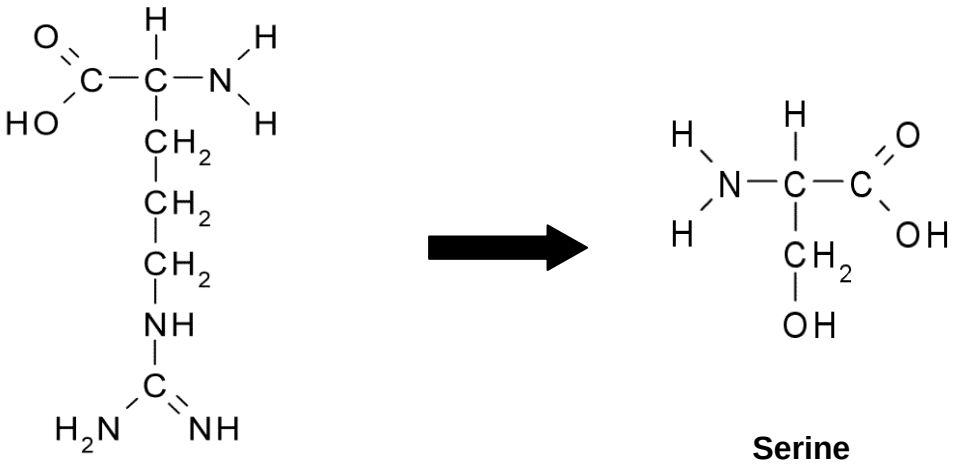
Fig. 33. Alignment of the amino acid sequences of BIS1 and BIS2

3.4.1. Single mutations of BIS1 toward BIS2

For both BISs, the preferred starter substrate is benzoyl-CoA and the second best starter is salicoyl-CoA (Table 3). The relative activity of BIS2 with salicoyl-CoA is somewhat higher than that of BIS1, raising the question as to the amino acids determining this difference. Therefore, point mutations in BIS1 toward BIS2 were

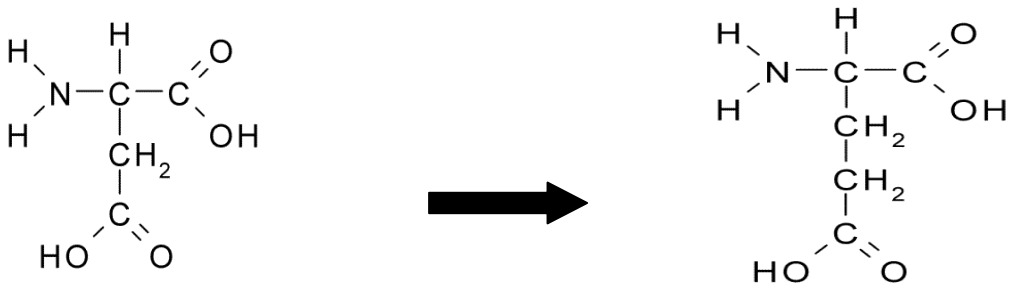
inserted at various positions and the substrate utilization profiles of the enzyme mutants were determined.

R231S : The amino acid at this position in BIS1 was arginine, a bulky basic residue, which was changed in the mutant to serine, a smaller polar amino acid.



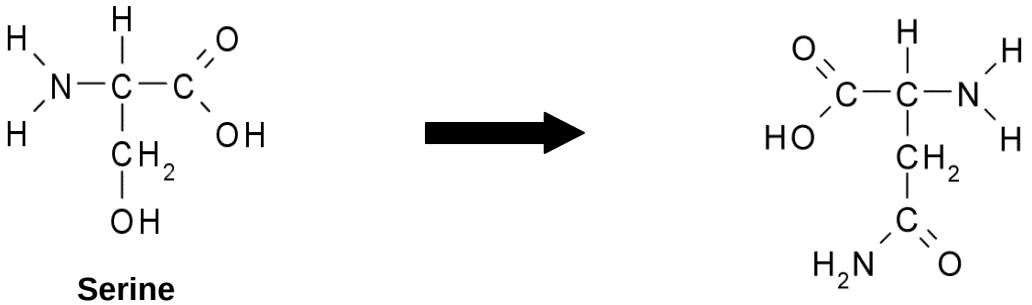
Arginine **Serine**

D267E : Aspartic acid, an acidic residue which resides at position 267 in BIS1, was changed to glutamic acid with a slightly larger side chain.



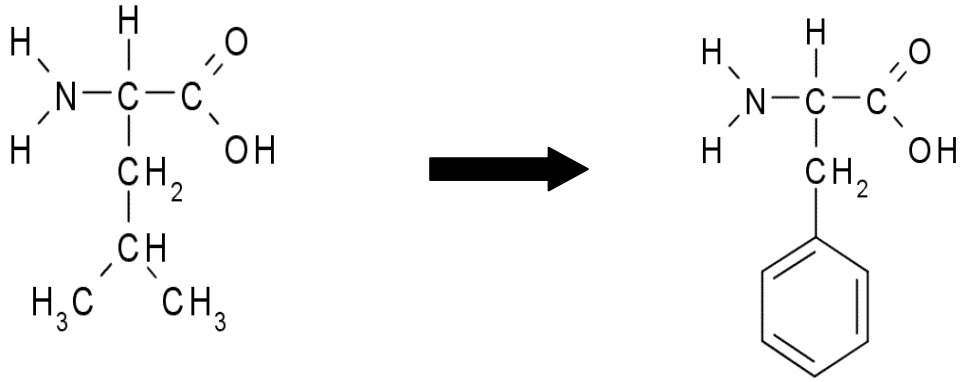
Aspartic acid **Glutamic acid**

S275N : The amino acid at this position in BIS1, a polar serine, was changed to another polar residue, asparagine.



Serine **Asparagine**

L299F : The fourth single mutation was performed at position 299 in BIS1. Leucine, an aliphatic amino acid, was changed to phenylalanine with an aromatic side chain.



Leucine **Phenylalanine**

The activities of the affinity-purified single mutants with various starter substrates were determined and compared with those of BIS1 (wild-type) and BIS2 (Table 5).

Table 5. Effect of point mutations in BIS1 toward BIS2 Product amount formed by mutant vs. product amounts formed by BIS1 wild-type/BIS2 wild-type)

	R231S	D267E	S275N	L299F
Benzoyl-CoA	As wild type	As wild type	3.4 ng vs. 1.47 ng / 2.54 ng	1.9 ng vs. 1.47 ng / 2.54 ng
o-Hydroxybenzoyl-CoA	As wild type	0.096 ng vs. 0.25 ng / 0.6 ng	1.2 ng vs. 0.25 ng / 0.6 ng	No activity
m- Hydroxybenzoyl-CoA	No activity	No activity	No activity	No activity
p-Hydroxybenzoyl-CoA	No activity	No activity	No activity	No activity
Cinnamoyl-CoA	No activity	No activity	No activity	No activity
Aliphatic-CoAs	No activity	No activity	No activity	No activity

3.4.2. Double mutation in BIS1 toward BIS2

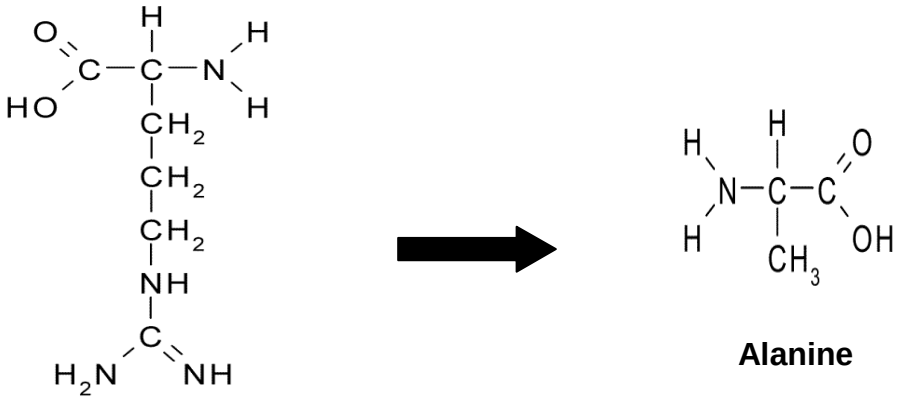
The mutant S275N shows an increase in the activity with salicoyl-CoA, while the activity with benzoyl-CoA resembles that of BIS2. In contrast, L299F had no longer detectable activity with salicoyl-CoA and similar activity with benzoyl-CoA as the wild-type. In the D267E mutant, the activity with salicoyl-CoA was somewhat reduced. These observations indicate that the two former positions have the strongest effect on substrate utilization. It was therefore interesting to generate a double mutation. At position 275, the amino acid was changed into asparagine and, in addition, the amino acid at position 299 was altered to phenylalanine. The result was an increase in the activities with both substrates, benzoyl-CoA and *o*-hydroxybenzoyl-CoA, which were higher than those exhibited by BIS1 and BIS2 (Table. 6).

Table. 6. Effect of a double mutation in BIS1 toward BIS2 (Product amount formed by double mutant vs. product amounts formed by BIS1 wild-type/BIS2 wild-type)

	S275N / L299F
Benzoyl-CoA	4.6 ng vs. 1.47 ng / 2.54 ng
o-Hydroxybenzoyl-CoA	1.56 ng vs. 0.25 ng / 0.6 ng
m- Hydroxybenzoyl-CoA	No activity
p-Hydroxybenzoyl-CoA	No activity
Cinnamoyl-CoA	No activity
Aliphatic-CoAs	No activity

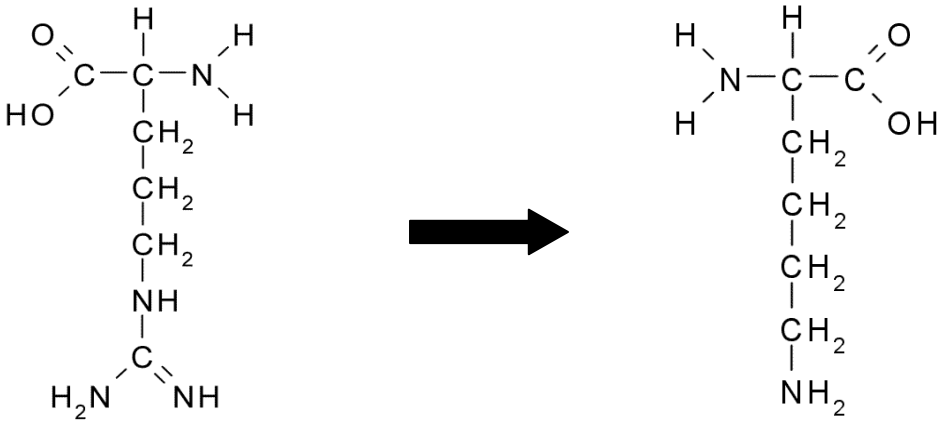
3.4.3. Single mutations of BIS1 independent of BIS2

R231A : The amino acid at this position in BIS1 was arginine (bulky, basic), which was changed to alanine with a relatively small hydrophobic side chain.



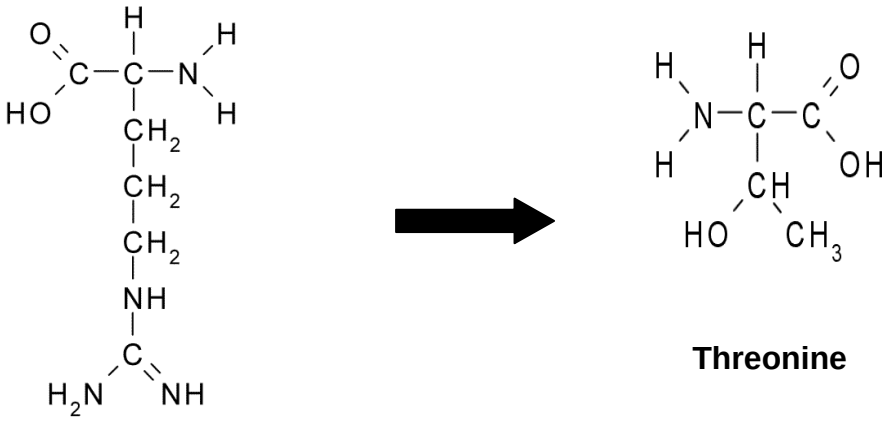
Arginine **Alanine**

R231K : The same position was mutated to lysine, a large basic amino acid.



Arginine **Lysine**

R231T : The third mutation at this position was a replacement with threonine, a polar amino acid.



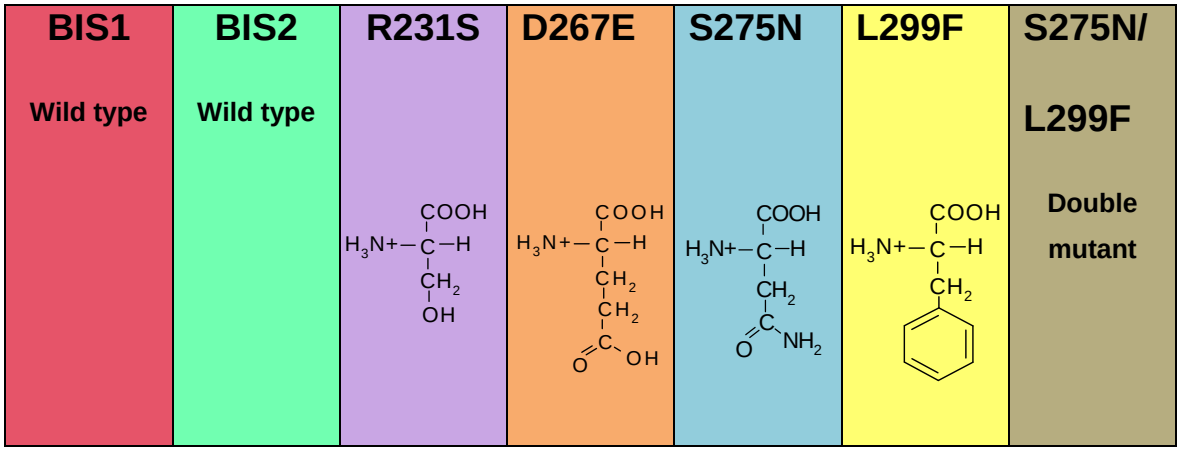
Arginine **Threonine**

The influence of the various mutations at position 231 is documented in Table 7

Table. 7. Effect of single mutations in BIS1 at position 231

	R231A	R231K	R231T
Benzoyl-CoA	Lower activity than BIS1 wild-type: 0.18 ng vs 1.47 ng	No activity	Lower activity than BIS1 wild-type: 0.34 ng vs 1.47 ng
o-Hydroxybenzoyl-CoA	No activity	No activity	No activity
m- Hydroxybenzoyl-CoA	No activity	No activity	No activity
p-Hydroxybenzoyl-CoA	No activity	No activity	No activity
Cinnamoyl-CoA	No activity	No activity	No activity
Aliphatic-CoAs	No activity	No activity	No activity

The BIS1 mutants that display interesting changes in substrate specificity are shown comparatively in Fig. 34. Most dramatic effects were observed with the single mutants S275N and L299F and the combination thereof as a double mutant.



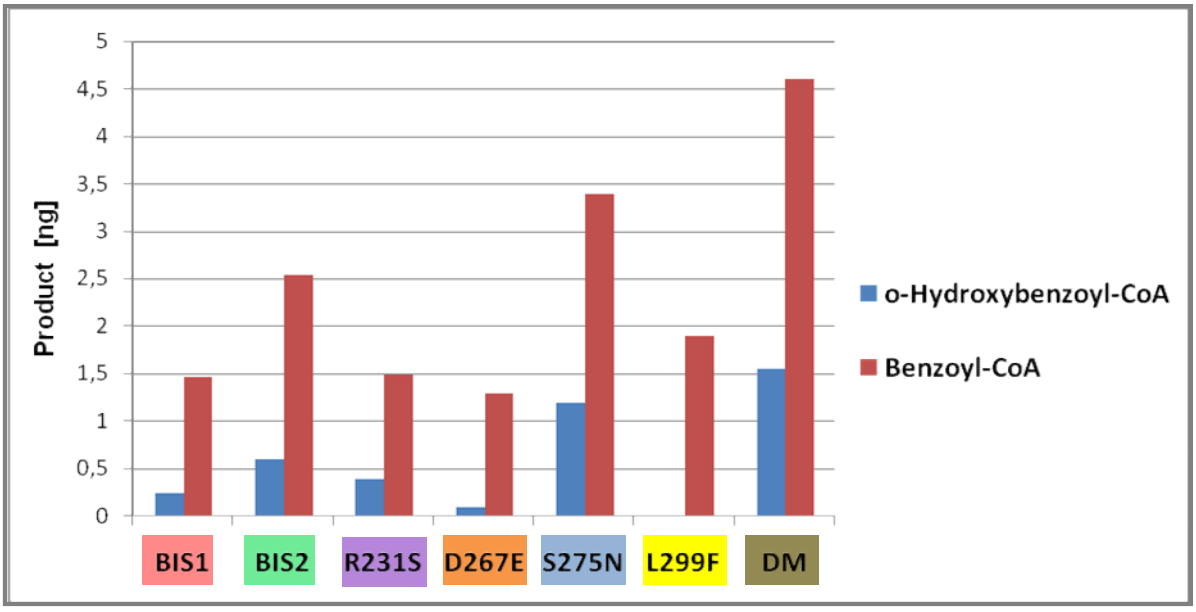


Fig. 34. BIS1 mutants exhibiting altered substrate specificities and catalytic activities

04 Discussion

4.1. Plant type III polyketide synthases (PKS)

A number of type III polyketide synthases (PKSs) have been found in plants generate an amazingly diverse array of secondary metabolites by catalyzing the sequential condensation of acetyl units derived from the malonyl-CoA thioester into a growing polyketide chain (Austin and Noel, 2003). This reaction sequence mirrors the biosynthetic pathway of the fatty acid synthase enzymes of primary metabolism, from which the PKSs likely evolved via gene duplication events and subsequent refunctionalization of the gene duplicates. The PKSs in plants constitute the type III PKS superfamily of enzymes, also called superfamily of CHS-like enzymes (Schröder, 2000; Austin and Noel, 2003). They utilize a variety of different starter substrates ranging from aliphatic-CoA to aromatic-CoA substrates, from small (acetyl-CoA) to bulky (p-coumaroyl-CoA) substrates or from polar (malonyl-CoA) to nonpolar (isovaleroyl-CoA) substrates giving the plants an extraordinarily functional and thereby chemical diversification (Flores-Sanchez and Verpoorte, 2009). This remarkable variety is further increased by divergences in the number of condensation reactions (polyketide chain elongation) and the type of the intramolecular cyclization reaction used (Schröder, 2000).

Examples of type III PKSs that participate in the biosynthesis of secondary metabolites are chalcone synthase (CHS), benzophenone synthase (BPS), biphenyl synthase (BIS) and biphenylcarboxylate synthase (BICS) (Table 8; Fig 35).
One of the aims of the present study was molecular cloning of biphenylcarboxylate synthase (BICS) from *G. lutea*. This enzyme is a specific plant type III PKS involved in the formation of amarogentin natural compounds as components of secondary metabolism in *G. lutea*. Plant remedies containing amarogentin are traditionally used as bitter tonics and stomachics. Beside these uses interesting novel pharmacological activities of amarogentin have been observed in recent years. The compound is a potent inhibitor of DNA topoisomerase I from *Leishmania donovani* (Ray et al, 1996,

and Kedzierski et al, 2009). In a hamster model of experimental leishmaniasis, amarogentin lacked toxicity (Medda et al, 1999) and might thus be a lead compound for designing effective antileishmanial drugs. In addition, amarogentin significantly inhibited the proliferation of cancer cells and induced apoptosis in a mouse skin carcinogenesis model (Saha et al, 2004, 2006).

Table 8. Selected examples of plant polyketide synthases and thier preferred substrates and released reaction products.

Enzyme	Substrates (starter, extender, number of condensations)	Type of ring closure	Products	Plant species	References
Chalcone synthase (CHS)	*p*-Coumaroyl-CoA, malonyl-CoA (3X)	Claisen, aromatic	Naringenin chalcone (1)	ubiquitous	(Austin and Noel, 2003)
Benzophenone synthase (BPS)	*m*-Hydroxybenzoyl-CoA, malonyl-CoA (3X) Benzoyl-CoA, malonyl-CoA (3X)	Claisen, aromatic	2,3´, 4,6-Tetrahydroxybenzophenone (2) 2,4,6-Trihydroxybenzophenone (3)	*Centaurium erythraea* *Hypericum androsaemum*	(Beerhues,1996) (Liu et al, 2003)
Biphenyl synthase (BIS)	Benzoyl-CoA, malonyl-CoA (3X)	Aldol, aromatic	3,5-Dihydroxybiphenyl (4)	*Sorbus aucuparia*	(Liu et al, 2007)
Biphenylcarboxylate synthase (BICS)	*m*-Hydroxybenzoyl-CoA, malonyl-CoA (3X)	Aldol without Decarbox-ylation, aromatic	3,3',5-Trihydroxybiphenyl-2-carboxylate in amarogentin (5)	*Gentiana lutea*	under study

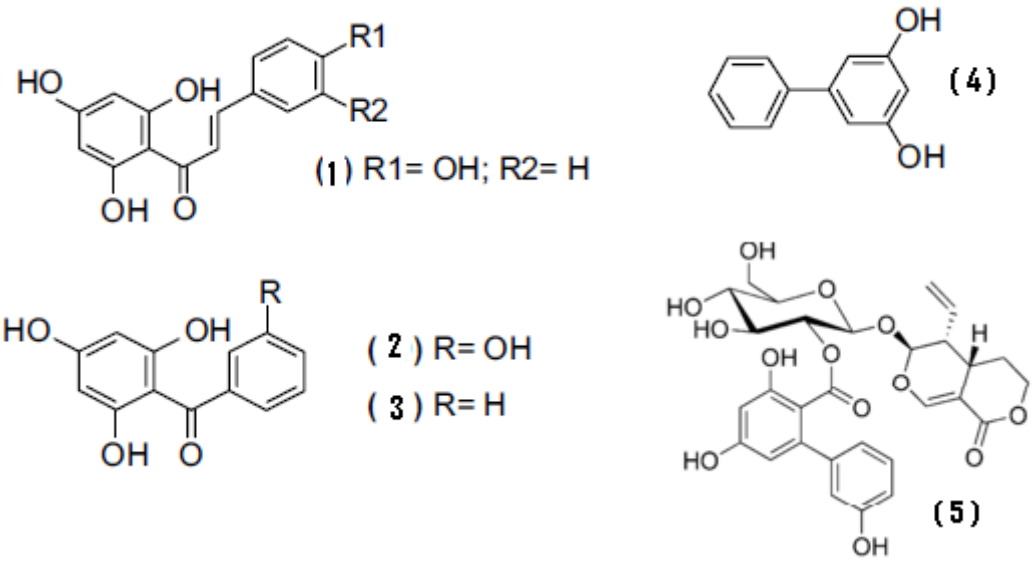


Fig. 35. Natural products biosynthesized by plant PKSs

4.1.1. BICS and CHS

The best known plant PKS is chalcone synthase (CHS; Table 8) which was both the first functionally and the first structurally characterized member of the superfamily (Schröder, 2000; Ferrer et al., 1999). CHS is a pivotal enzyme in the biosynthesis of flavonoid antimicrobial phytoalexins and anthocyanin pigments in plants (Abe et al, 2005; Ferrer et al., 1999). Anthocyanins, a colored class of flavonoids, are major constituents of flower and fruit colors. The majority of the naturally occurring flavonoids are glycosylated with one or more sugar groups. The best-studied flavonoid glucosyltransferase is UDP-Glc:flavonoid 3-O-glucosyltransferases (3GT). The 3GT enzyme has been characterized biochemically and molecular biologically in gentian (Tanaka et al., 1996). Flavonoid biosynthesis starts with general phenylpropanoid metabolism. The first committed step is catalyzed by chalcone synthase (CHS), which involves the sequential condensation of one *p*-coumaroyl-CoA and three malonyl-CoA molecules (Winkel-Shirley, 2001). After initial capture of the *p*-coumaroyl moiety, each subsequent condensation step begins with decarboxylation of malonyl-CoA at the CHS active site; the resulting acetyl-CoA carbanion then serves as the nucleophile for chain elongation. Ultimately, these reactions generate a tetraketide intermediate that cyclizes by Claisen condensation into a hydroxylated aromatic ring system (Ferrer et al., 1999). CHS from *Medicago*

sativa was crystallized and clarified in its spatial structure (Ferrer et al., 1999). In the present work, a near full-length cDNA (1343 bp) and a partial cDNA (741 bp) encoding PKSs were isolated. Clone (1) exhibits about 83 % amino acid / nucleotide sequence identity with CHS and may thus encode this enzyme in gentian. Clone (2) exhibits only 73 % identity with CHS and may code for BICS.

4.1.2. BICS and BPS

BPS is the key enzyme of xanthone biosynthesis. The majority of natural xanthones have been found in just two families of higher plants, Guttiferae and Gentianaceae. Oxygenated xanthones occur in both families and are generally more highly oxygenated in the Gentianaceae, whereas prenylated xanthones are widely distributed in the Guttiferae but not known in the Gentianaceae (Bennette and Lee, 1989). The carbon skeleton of benzophenone derivatives is formed by BPS. The enzyme from *Hypericum androsaemum* (Guttiferae) catalyzes the iterative condensation of benzoyl-CoA with three molecules of malonyl-CoA to give a linear tetraketide intermediate which is subsequently cyclized into 2,4,6-trihydroxybenzophenone via intramolecular Claisen condensation (Liu et al, 2003). In contrast BPS from *Centaurium erythraea* (Gentianaceae) prefers 3-hydroxybenzoyl-CoA as a starter substrate, yielding 2,3',4,6 tetrahydroxybenzophenone (Beerhues, 1996). These two BPS products are the precursors of all prenylated benzoylphloroglucinols and xanthones. The functional behavior of *H. androsaemum* BPS was dramatically altered by a single amino acid substitution in the active site cavity, which transformed BPS into phenylpyrone synthase (PPS; Klundt et al, 2009). The point mutation modulated between the formation of a tetraketide product derived from Claisen condensation and a triketide product derived from lactone formation. A common and interesting feature of polyketides occurring in the Gentianaceae is that a partial structure is derived from 3-hydroxybenzoic acid (Abd El-Mawala et al, 2001; Wang et al, 2003). 3-Hydroxybenzoic acid is directly derived from the shikimic acid pathway, whereas benzoic acid originates from cinnamic acid by side-chain degradation (Abd El-Mawla et al., 2001; Abd El-Mawla and Beerhues, 2002; Wang et al., 2003). Thus, BPS and BICS use 3-hydroxybenzoyl-CoA as the starter substrate along with malonyl-CoA as the extender substrate. When the two newly isolated

cDNA clones from *G. lutea* were compared with the BPS sequences so far available, no appreciable sequence similarities were observed.

4.1.3. BICS and BIS

BIS is a recently detected type III PKS, which is responsible for the biosynthesis of the C_{12} skeleton of biphenyls and related dibenzofurans (Liu et al, 2004). These two clases of compounds are the phytoalexins of the Maloideae (Kokubun and Harborne, 1995). In yeast-extract-treated cell cultures of *S. aucuparia*, a massive accumulation of aucuparin was detected, which is the best known biphenyl phytoalexin (Liu et al, 2004). The starter substrate for BIS is, as for BPS, benzoyl-CoA which in general is a rare starter molecule for PKSs. BIS catalyzes the iterative condensation of benzoyl-CoA with three acetyl units from malonyl-CoA to give a linear tetraketide, which undergoes an intramolecular C2 ⟶ C7 aldol condensation and decarboxylative elimination of the terminal carboxyl group (Liu et al, 2007). Compared to BIS, BICS is likely to catalyze a similar reaction mechanism, with the exception that the terminal carboxyl group is not eliminated but remains in the molecule (Fig. 5). Furthermore, BICS uses 3-hydroxybenzoyl-CoA as a starter substrate instead of benzoyl-CoA which is preferred by BIS. Suprisingly, the two cDNA sequences cloned from *G. lutea* lacked similarity with BISs. In summary, the new clones shared 73 - 83% identity with CHS but exhibited similarity neither with BPS nor with BIS. The future efforts have to be directed toward the cloning of the full-length sequences for heterologous expression and functional analysis.

4.2. BISs from *P. communis*

For the biochemical characterization of the recombinant BISs, the proteins were used immediately after purification on Ni-NTA agarose. Storage of the enzymes at -20°C was not possible because their activity significantly decreased after freezing, independent of the presence or absence of glycerol.

BISs from *P. communis* and BIS1 from *S. aucuparia* share around 96 % amino acid sequence identity. Functionally divergent plant type III PKSs usually share 60 – 95 % identity with each other (Austin and Noel, 2003). For both pear BISs, benzoyl-CoA is implemented as the preferred starter substrate and 3,5-dihydroxybiphenyl is the

reaction product. Beside benzoyl-CoA, the two recombinant enzymes also accept *o*-hydroxybenzoyl-CoA as a starter substrate; however, the relative activities were lower. Instead of the corresponding biphenyl, the enzymatic product resulted from only one condensation reaction to give 4-hydroxycoumarin, which was previously detected by Liu et al, (2010). The apparent K_m values, as determined from Lineweaver-Burk plots under optimum conditions, were 1.6 and 1.4 µM (benzoyl-CoA) and 6.5 and 17.1 µM (malonyl-CoA) for BIS1 and BIS2, respectively. The kinetic parameters determined for the BISs from *P. communis* are correlated with the values for the BISs from *S. aucuparia* (Table 9). Both the K_m and K_{cat} values obtained with benzoyl-CoA are similar between the enzymes from the two species.

Table 9. Kinetic parameters for BISs from *P. communis* and *S. aucuparia*

		P. communis		***S. aucuparia*** (Liu et al, 2007, 2010)		
Substrate Parameter		BIS1	BIS2	BIS1	BIS2	BIS3
Benzoyl-CoA	K_m(µM)	1.6	1.7	0.7	1.4	1.6
	K_{cat} (min^{-1})	0.65	0.74	0.43	0.89	1.02
	K_{cat}/K_m ($M^{-1}s^{-1}$)	6771	7255	10181	10987	10625
o-Hydroxybenzoyl-CoA	K_m(µM)	Not determined	Not determined	0.80	2.3	3.2
	K_{cat} (min^{-1})	Not determined	Not determined	0.27	1.76	2.22
	K_{cat}/K_m ($M^{-1}s^{-1}$)	Not determined	Not determined	5625	12716	11486
Malonyl-CoA	K_m(µM)	6.5	17.1	6.2	10.0	10.1

BIS1 mRNA in *P. communis* leaves was detected 2 and 6 days after *E. amylovora* infection but BIS2 mRNA was not. A more detailed study of the time course of the BIS1 mRNA accumulation will be conducted in the future. A differential expression of BIS isoforms has recently also been observed in *S. aucuparia* (Liu et al. 2010). The maximum BIS3 transcript level was observed around 6 h after the onset of elicitation and was lower than the maximum BIS1 mRNA level at 9h. In contrast to BIS1 and BIS3 transcripts, BIS2 mRNA was not induced by elicitor treatment (Liu et al, 2010). Following fungal infection, biphenyls and dibenzofurans accumulate as phytoalexins in the sapwood of a number of Maloideae species (Kokubun and Harborne, 1995). The antifungal activity of biphenyls and dibenzofurans is due to inhibition of both spore germination and mycelial growth at concentrations thought to be present at localized infection sites (Hrazdina et al, 1997). *Pyrus* species were found to form dibenzofurans, such as pyrufurans, and to lack biphenyls (Kokubun and Harborne 1995). In contrast, *Malus* was described as a biphenyl producer. However, cell cultures of a scab-resistant cultivar produced phytoalexins in response to yeast extract treatment, with malusfuran being a prominent component (Hrazdina et al, 1997). Yeast-extract treated *M. domestica* leaves (in vitro propagated plants) failed to produce biphenyl and dibenzofuran phytoalexins (Hrazdina, 2003). *S. aucuparia* is an exception because here the leaves, and not the sapwood, accumulate the biphenyl aucuparin in response to biotic and abiotic elicitation (Kokubun and Harborne, 1994).
The second best starter substrate for the BISs from *P. communis* was salicoyl-CoA. The enzymes catalyzed a single decarboxylative condensation reaction with malonyl-CoA to form a diketide intermediate which underwent intramolecular cyclization by nucleophilic attack of the phenol group on the CoA- or cysteine-tethered C-1 thioester, yielding 4-hydroxycoumarin after enolization (Fig. 36; Liu et al, 2010). The cysteine residue belongs to the catalytic triad conserved among type III PKSs (Jez et al, 2002). When elicitor-treated *S. aucuparia* cell cultures were fed with the N-acetylcysteamine (NAC) thioester of salicylic acid, 4-hydroxycoumarin accumulated in the culture medium. However 4-hydroxycoumarin was not detectable in elicitor-treated *S. aucuparia* cell cultures without feeding salicoyl-CoA, indicating that salicoyl-CoA is not available as an endogenous starter substrate for the BIS isoenzymes (Liu et al, 2010).

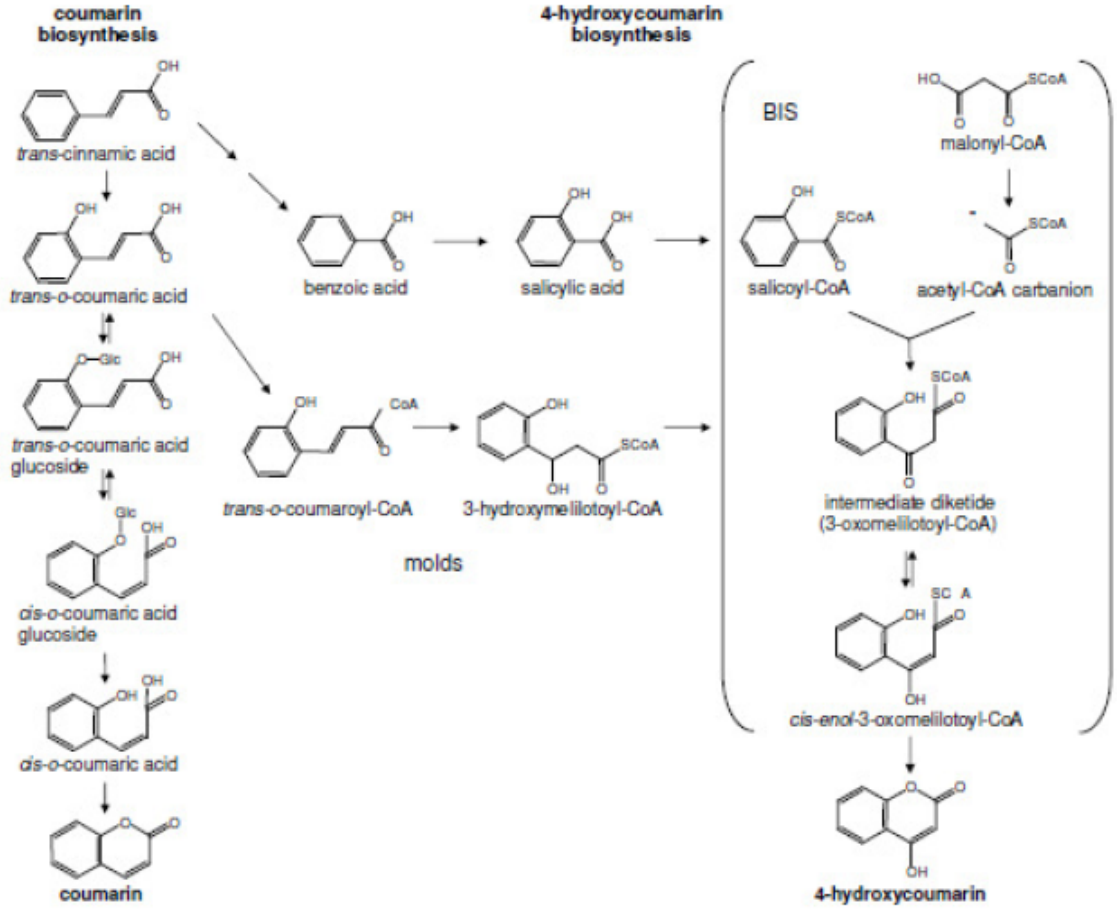


Fig. 36. Proposed biosynthetic pathways of coumarin and 4-hydroxycoumarin.

4-Hydroxycoumarin is one of the derivatives of the well-known coumarin molecule (Fig. 37, A). Coumarin derivatives are widely distributed in higher plants where they originate from the general phenylpropanoid pathway. Coumarin itself is a natural product famous for its pleasant vanilla-like odor (Bourgaud et al, 2006).

Biosynthesis of 4-hydroxycoumarin (Fig. 37, B) is commonly attributed to the action of molds, such as *Penicillium* and *Aspergillus* species, on melilotoside-containing plants, such as sweet clover (*Melilotus alba,* Fabaceae). These occasionally undergo spoilage during storage, associated with moulding and 'fermentation' of the hay. Both melilotic acid (*o*-hydroxyphenylpropionic acid) and *o*-coumaric acid can serve as precursors for dicoumarol (Fig. 37, C) production. 4-Hydroxycoumarin can react non-enzymatically with formaldehyde to form dicoumarol (Bye and King, 1970).

Dicoumarol is well-known for its pronounced blood anticoagulant properties which can cause the deaths of livestock by internal bleeding (sweet clover disease) and is the forerunner of the warfarin (Fig. 37, D) group of medicinal anticoagulants (Liu et al, 2010).

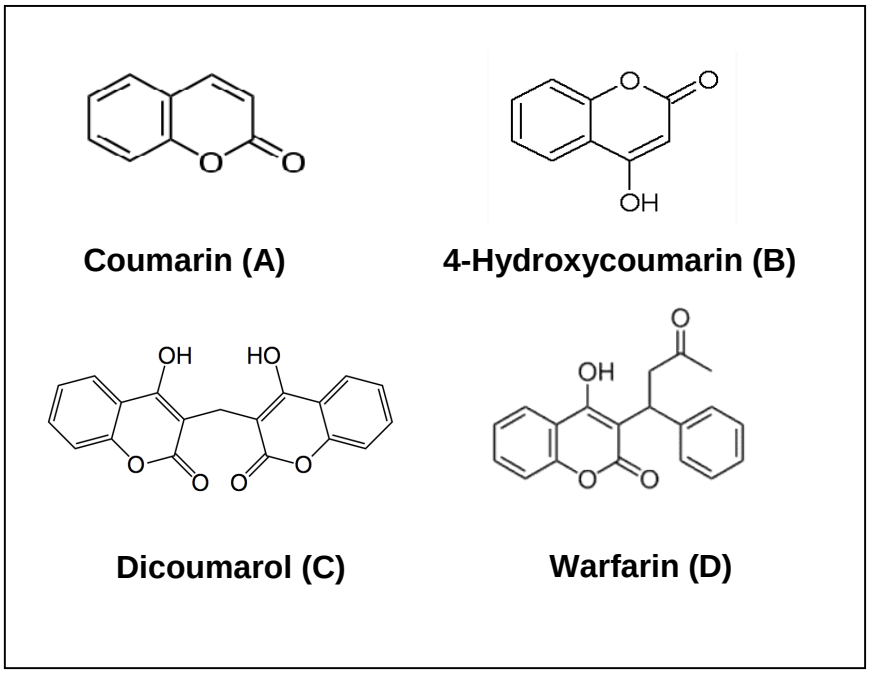


Fig. 37. Structural formulae of natural and medicinal coumarin derivatives

4.3. Site-directed mutagenesis of BIS1

A site-directed mutant of BIS1, S275N (278 in *M. sativa* CHS numbering) exhibited enhanced catalytic activity with both benzoyl-CoA and salicoyl-CoA, while the L299F substitution (300 in *M. sativa* CHS numbering) abolished the activity with salicoyl-CoA. The double mutant S275N/L299F possessed strongly increased activity with both starter substrates. Protein homology modeling will be useful in the future to explain the significant modifications observed in the specificities of the mutants created. The other point mutations generated (R231S, D267E, R231A, R231K and R231T; corresponding to R234S, D270E, R234A, R234K and R234T, respectively, in *M. sativa* CHS numbering) showed no dramatic change in substrate or product specificities. However, a more or less pronounced reduction in the catalytic activities was observed.

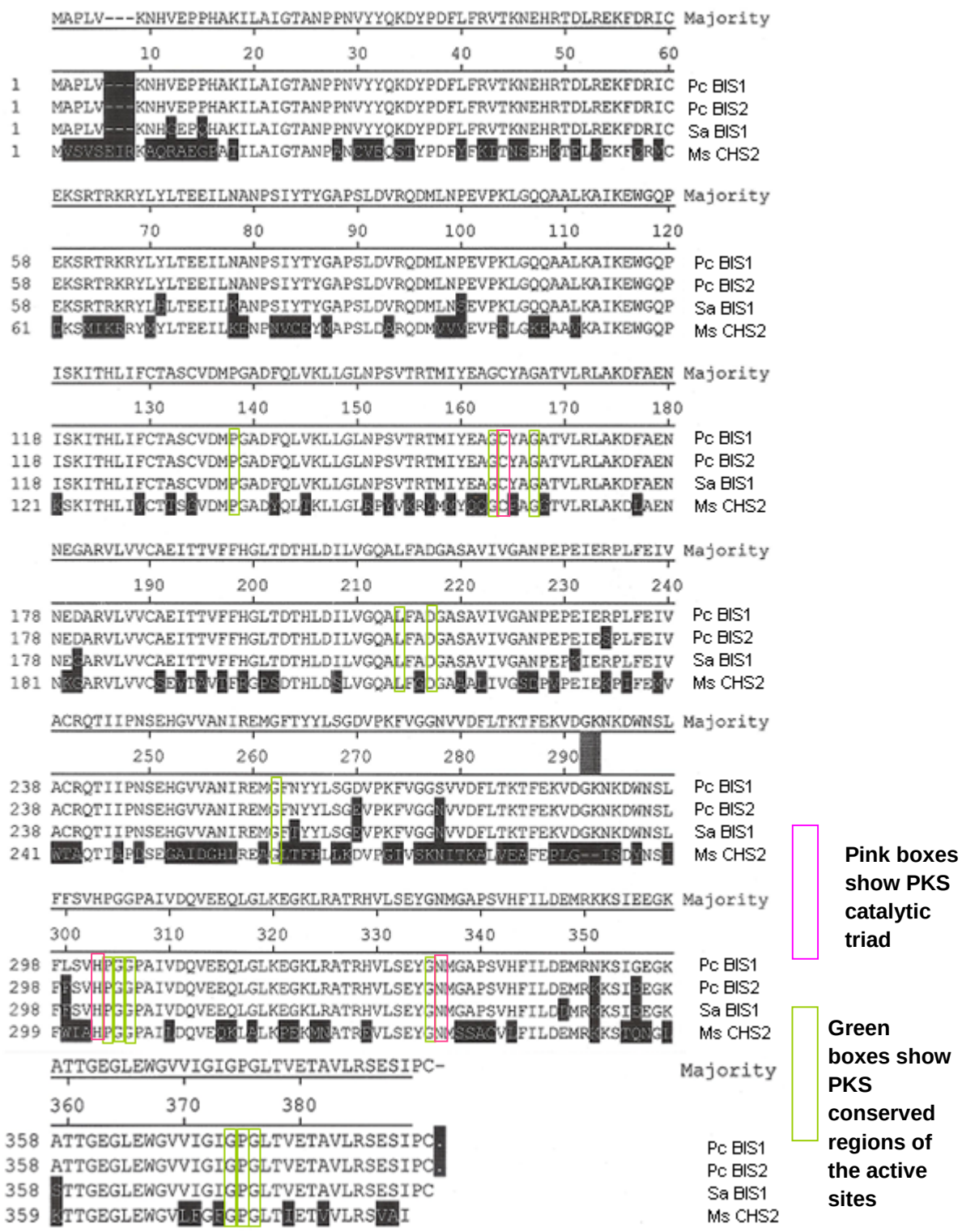


Fig. 38. Alignment of the amino acid sequences of BISs from *P. communis*, BIS1 from *S. aucuparia* and CHS from *M. sativa*

All CHS-like proteins exihibt strong conservation of residues shaping the geometry of the active site (Pro 138, Gly 163, Gly 167, Leu 214, Asp 217, Gly 262, Pro 304, Gly 305, Gly 306, Gly 335, Gly 374, Pro 375 and Gly 376; Fig. 38). In addition, each type III PKS monomer utilizes a Cys 164 - His 303 - Asn 336 catalytic triad within a buried active site cavity, as first revealed in the crystal structure of alfalfa CHS (Ferrer et al, 1999).

Site-directed mutations that alter the substrate and/or product specificities of type III PKSs were previously reported. Mutation of Phe-265 to valine, as found in acridone synthase (ACS), does not alter the starter molecule preference of alfalfa CHS, but the F215S CHS mutant accepts *N*-methylanthraniloyl-CoA as a substrate and yields a novel alkaloid, namely *N*-methylanthraniloyltriacetic acid lactone (Jez et al, 2002). The naturally occurring acridone is not formed. Comparison of the three-dimensional structures of wild-type CHS and the F215S mutant suggests that substitution of a serine for Phe-215 allows for productive binding of *N*-methylanthraniloyl-CoA by allowing the bulkier starter molecule to reside in the wider active site entrance (Jez et al, 2002).

Aloesone synthase (ALS) from *Rheum palmatum* is the first plant-specific type III PKS that catalyses formation of a heptaketide, aloesone, by accepting acetyl-CoA as a starter to carry out six successive condensations with malonyl-CoA (Abe et al, 2006). Steric modulation of the active site cavity demonstrated that three residues, Ala197, Leu256, and Thr338, are crucial for the remarkable catalytic activity of *R. palmatum* ALS. When Leu256 was substituted with less bulky Gly, the resulting L256G mutant accepted 4-coumaryl-CoA as a starter to efficiently produce 4-coumaroyltriacetic acid lactone (CTAL) as a derailment product. The wild-type enzyme did not accept the coumaroyl-CoA starter (Abe et al, 2006).

The functional behavior of BPS was dramatically altered by a single amino acid substitution in the active site cavity, which transformed BPS into PPS. The point mutation modulated between the formations of a tetraketide product derived from Claisen condensation and a triketide product derived from lactone formation (Klundt et al, 2009). Replacement of the polar residue Thr135 of BPS with the apolar amino acid leucine in PPS may open the gate to a buried pocket, into which the triketide intermediate is redirected during the elongation process of the diketide. The size of the newly accessible pocket in PPS compared with BPS is smaller and does not

allow for a third extension with a malonyl-CoA (Klundt et al, 2009). 3-Hydroxybenzoyl-CoA is the second best starter substrate for wild type BPS but a poor starter molecule for PPS. In this enzyme mutant, the 3-hydroxybenzoyl-primed triketide may be trapped in the new pocket by hydrogen bonds of the 3-hydroxyl group with the backbone, thereby acting as an inihibitor (Klundt et al, 2009).

Although 2-pyrone synthase (2-PS) and CHS share 74% amino acid sequence identity, 2-PS forms a triketide from an acetyl-CoA starter molecule and two malonyl-CoAs, which then cyclizes into 6-methyl-4-hydroxy-2-pyrone (Eckermann et al, 1998). CHS forms a tetraketide using a *p*-coumaroyl-CoA starter molecule and three malonyl-CoAs, which then cyclizes into 4,2´,4´,6´-tetrahydroxychalcone. Comparison of the three-dimensional structures of 2-PS and CHS reveals that the volumes of the internal cavities that accommodate the growing polyketid are dramatically different. The 2-PS cavity is much smaller than the CHS cavity. Of 28 residues lining the 2-PS initiation/elongation cavity, four positions vary in CHS. Point mutations at three of these positions in CHS (T197L, G256L, S338I) yielded an enzyme that was functionally identical to 2-PS. Thus, the shape and the volume of the active site cavity greatly influence the substrate specificity and control the final length of the polyketide (Jez et al, 2000).

The catalytic triad of *M. sativa* CHS and a Phe residue (Cys164, Phe215, His303, Asn336) are well conserved in all the known type III PKSs. The only exception is the benzalacetone synthase (BAS) of *Rheum palmatum*, which lacks the active site residue Phe215 and has a Leu at this position (Abe et al, 2001). Phe215 has been proposed to facilitate malonyl-CoA decarboxylation and help orient substrates and intermediates during the sequential condensation reactions in CHS (Jez et al, 2000). This is the reason why in BAS the polyketide chain elongation reaction is terminated at the diketide stage (Abe et al, 2002).

The crystal structure of *M. sativa* CHS alone and complexed with a series of substrate and product analogs provided a framework for understanding the biosynthesis of plant polyketides and revealed how a single enzyme active site orchestrates a series of decarboxylation and condensation reactions and how it controls the stereochemistry of the cyclization reaction leading to the formation of structurally complex and biologically important natural products. This information combined with the sequence data available allowed identification of plant PKSs

possessing new substrate and product specificities. In addition, CHS-like enzymes, modified by mutagenesis, can be used to engineer biosynthetic pathways for the production of novel compounds (Ferrer et al, 1999).

05 Perspective studies

Ø *G. lutea* and several related gentians are endangered species in most European countries; however attempts to cultivate *G. lutea* were met with difficulties. Micropropagation is therefore an alternative cultivation system and protocols for *in vitro* tissue cultures or plants have to be elaborated, which may replace a natural source of *G. lutea*.

Ø Further attempts of 5´cloning to get the two full-length clones from *G. lutea* have to be made, followed by heterologous expression of the recombinant enzymes in *E. coli*, their purification by affinity-chromatography and characterization of their structural and kinetic properties.

Ø The results obtained by site-directed mutagenesis of pear BIS1 need to be rationalized by means of protein homology modeling to gain deeper insight into structure-function relationships.

06 SUMMARY

Ø *Gentiana lutea* (Gentianaceae) is a herbaceous medicinal plant containing amarogentin. It is traditionally used for preparation of bitter tonics and stomachics. In recent years, interesting novel pharmacological activities of amarogentin have been observed, such as inhibition of DNA topoisomerase I from *Leishmania donovani*. It also inhibits proliferation of cancer cells and induces apoptosis in a mouse skin carcinogenesis model.

Ø A partial structure of amarogentin is an unusual 3,3',5-trihydroxybiphenyl-2-carboxylate moiety. The biosynthesis of this intermediate was postulated to be catalyzed by a type III polyketide synthase (PKS), called biphenylcarboxylate synthase (BICS). In comparison, biphenyl synthase (BIS) is responsible for the formation of biphenyls lacking the 2-carboxyl group. Biphenyls are phytoalexins of the Rosaceous subfamily Maloideae, which includes important fruit trees such as apple and pear.

Ø One goal of this work was molecular cloning of a BICS cDNA from *G. lutea*. Two cDNA fragments were obtained starting with degenerate primers derived from conserved regions of type III PKSs. The clones were 1343 and 741 bp long and shared 73 - 83% identity with chalcone synthase (CHS) but lacked appreciable identity with BIS and benzophenone synthase (BPS).

Ø In addition, two BIS cDNAs were cloned from young leaves of *P. communis* using a primer pair derived from the non-coding regions of *S. aucuparia* BIS. The two pear BISs share 98.2% amino acid sequence identity.

Summary

Ø The BIS enzymes were functionally expressed in *E. coli.* Both enzymes preferred benzoyl-CoA as a starter substrate and formed 3,5-dihydroxybiphenyl. With salicoyl-CoA, the enzymatic product was 4-hydroxycoumarin. *m*- and *p*-Hydroxybenzoyl-CoAs and *p*-coumaroyl-CoA were not accepted as starters.

Ø The kinetic properties of the pear BISs were similar to those observed for the BISs from *S. aucuparia*. For the preferred starter substrate, benzoyl-CoA, the K_m values were 1.6 and 1.7 µM and the K_{cat} values (min^{-1}) were 0.65 and 0.74 for BIS1 and BIS2, respectively. For malonyl-CoA involved in the reaction as extender the K_m values were 6.5 and 17.1 µM for BIS1 and BIS2, respectively. Both BISs showed a pH optimum between 7 and 7.5 and a temperature optimum at 25 to 30˚C.

Ø Expression of the BISs was studied in leaves of *P. communis* after infection with *Erwinia amylovora*, the causative agent of fire blight. While BIS1 transcripts accumulated after infection, as shown by RT-PCR, expression of BIS2 was not induced.

Ø Six residues differ between the BIS1 and BIS2 amino acid sequences and were altered in BIS1 by site-directed mutagenesis. The S275N mutation enhanced the catalytic activity with both benzoyl-CoA and salicoyl-CoA as starters, while the L299F substitution abolished the activity with salicoyl-CoA. The double mutant S275N/L299F exhibited strongly increased activity with both starter substrates.

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